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(52) UK CL (Edition N)
A5B BHA B180 B42Y B422 B44Y B442 B45Y B450 B451 B48Y B482 B49Y B491 B50Y B502 B51Y B511 B54Y B540 B55Y B552 B56Y B565 B57Y B574 B58Y B585 B586 B59Y B596 B65Y B652 B653 B656 B66Y B663 C6Y Y125 Y406 Y407 Y410 Y419 Y501 Y503 U1S S1068 S1334 S1357 S2416

(56) Documents Cited
GB 2264948 A WO 93/25677 A1
US Pat.Appl. NTIS US 7-577528 Life Sci. 1993,52,1917-1924 Biochem.Biophys.Res.Commun. 1192,187(1),86-93 Mol.Endocrinol. 1992,6,384-393

(58) Field of Search
UK CL (Edition N) A5B BHA
INT CL^6 A61K 31/52
ONLINE: WPI,CLAIMS,DIALOG/BIOTECH

(54) **Inhibition of TNFalpha production with agonists of the A2b subtype of the adenosine receptor**

(57) TNFα production is inhibited by contacting the A2b subtype of the adenosine receptor with an adenosine receptor agonist, especially in monocytes in which cAMP accumulation is increased due to activation of adenylate cyclase. The agonist is preferably adenosine 5'-(N-cyclopropyl)carboxamidoadenosine, 5'-(N-ethyl)carboxamideadenosine, (R)-N^6-phenyl-2-propyladenosine or cyclohexyladenosine. The agonists may be used in the therapy of autoimmune states. A process for the identification of A2b adenosine receptor agonist, or selective, compounds is described, involving treating monocytes with the compound to determine the degree of TNFα inhibitor, and selecting those compounds which either bind specifically to the A2b adenosine receptor or which include cAMP increase in a cell line expressing the receptor.

GB 2 289 218 A

At least one drawing originally filed was informal and the print reproduced here is taken from a later filed formal copy.

10 20
Met Pro Pro Ser Ile Ser Ala Phe Gln Ala Ala Tyr Ile Gly Ile Glu Val Leu Ile Ala
30 40
Leu Val Ser Val Pro Gly Asn Val Leu Val Ile Trp Ala Val Lys Val Asn Gln Ala Leu
50 60
Arg Asp Ala Thr Phe Cys Phe Ile Val Ser Leu Ala Val Ala Asp Val Ala Val Gly Ala
70 80
Leu Val Ile Pro Leu Ala Ile Leu Ile Asn Ile Gly Pro Gln Thr Tyr Phe His Thr Cys
90 100
Leu Met Val Ala Cys Pro Val Leu Ile Leu Thr Gln Ser Ser Ile Leu Ala Leu Leu Ala
110 120
Ile Ala Val Asp Arg Tyr Leu Arg Val Lys Ile Pro Leu Arg Tyr Lys Met Val Val Thr
130 140
Pro Arg Arg Ala Ala Val Ala Ile Ala Gly Cys Trp Ile Leu Ser Phe Val Val Gly Leu
150 160
Thr Pro Met Phe Gly Trp Asn Asn Leu Ser Ala Val Glu Arg Ala Trp Ala Ala Asn Gly
170 180
Ser Met Gly Glu Pro Val Ile Lys Cys Glu Phe Glu Lys Val Ile Ser Met Glu Tyr Met
190 200
Val Tyr Phe Asn Phe Phe Val Trp Val Leu Pro Pro Leu Leu Leu Met Val Leu Ile Tyr
210 220
Leu Glu Val Phe Tyr Leu Ile Arg Lys Gln Leu Asn Lys Lys Val Ser Ala Ser Ser Gly
230 240
Asp Pro Gln Lys Tyr Tyr Gly Lys Glu Leu Lys Ile Ala Lys Ser Leu Ala Leu Ile Leu
250 260
Phe Leu Phe Ala Leu Ser Trp Leu Pro Leu His Ile Leu Asn Cys Ile Thr Leu Phe Cys
270 280
Pro Ser Cys His Lys Pro Ser Ile Leu Thr Tyr Ile Ala Ile Phe Leu Thr His Gly Asn
290 300
Ser Ala Met Asn Pro Ile Val Tyr Ala Phe Arg Ile Gln Lys Phe Arg Val Thr Phe Leu
310 320
Lys Ile Trp Asn Asp His Phe Arg Cys Gln Pro Ala Pro Pro Ile Asp Glu Asp Leu Pro
326
Glu Glu Arg Pro Asp Asp

FIG. 1

```
        10                  30                  50
atgccgccct ccatctcagc tttccaggcc gcctacatcg gcatcgaggt gctcatcgcc
        70                  90                 110
ctggtctctg tgcccgggaa cgtgctggtg atctgggcgg tgaaggtgaa ccaggcgctg
       130                 150                 170
cgggatgcca ccttctgctt catcgtgtcg ctggcggtgg ctgatgtggc cgtgggtgcc
       190                 210                 230
ctggtcatcc ccctcgccat cctcatcaac attgggccac agacctactt ccacacctgc
       250                 270                 290
ctcatggttg cctgtccggt cctcatcctc acccagagct ccatcctggc cctgctggca
       310                 330                 350
attgctgtgg accgctacct ccgggtcaag atccctctcc ggtacaagat ggtggtgacc
       370                 390                 410
ccccggaggg cggcggtggc catagccggc tgctggatcc tctccttcgt ggtgggactg
       430                 450                 470
acccctatgt ttggctggaa caatctgagt gcggtggagc gggcctgggc agccaacggc
       490                 510                 530
agcatggggg agcccgtgat caagtgcgag ttcgagaagg tcatcagcat ggagtacatg
       550                 570                 590
gtctacttca acttctttgt gtgggtgctg cccccgcttc tcctcatggt cctcatctac
       610                 630                 650
ctggaggtct tctacctaat ccgcaagcag ctcaacaaga aggtgtcggc ctcctccggc
```

FIG. 2A

```
       670                     690                     710
gacccgcaga agtactatgg gaaggagctg aagatcgcca agtcgctggc cctcatcctc
       730                     750                     770
ttcctctttg ccctcagctg gctgcctttg cacatcctca actgcatcac cctcttctgc
       790                     810                     830
ccgtcctgcc acaagcccag catccttacc tacattgcca tcttcctcac gcacggcaac
       850                     870                     890
tcggccatga accccattgt ctatgccttc cgcatccaga agttccgcgt caccttcctt
       910                     930                     950
aagatttgga atgaccattt ccgctgccag cctgcacctc ccattgacga ggatctccca
       970
gaagagaggc ctgatgacta g
```

F I G. 2B

10 20
Met Pro Ile Met Gly Ser Ser Val Tyr Ile Thr Val Glu Leu Ala Ile Ala Val Leu Ala
30 40
Ile Leu Gly Asn Val Leu Val Cys Trp Ala Val Trp Leu Asn Ser Asn Leu Gln Asn Val
50 60
Thr Asn Tyr Phe Val Val Ser Leu Ala Ala Ala Asp Ile Ala Val Gly Val Leu Ala Ile
70 80
Pro Phe Ala Ile Thr Ile Ser Thr Gly Phe Cys Ala Ala Cys His Gly Cys Leu Phe Ile
90 100
Ala Cys Phe Val Leu Val Leu Thr Gln Ser Ser Ile Phe Ser Leu Leu Ala Ile Ala Ile
110 120
Asp Arg Tyr Ile Ala Ile Arg Ile Pro Leu Arg Tyr Asn Gly Leu Val Thr Gly Thr Arg
130 140
Ala Lys Gly Ile Ile Ala Ile Cys Trp Val Leu Ser Phe Ala Ile Gly Leu Thr Pro Met
150 160
Leu Gly Trp Asn Asn Cys Gly Gln Pro Lys Glu Gly Lys Asn His Ser Gln Gly Cys Gly
170 180
Glu Gly Gln Val Ala Cys Leu Phe Glu Asp Val Val Pro Met Asn Tyr Met Val Tyr Phe
190 200
Asn Phe Phe Ala Cys Val Leu Val Pro Leu Leu Leu Met Leu Gly Val Tyr Leu Arg Ile
210 220
Phe Leu Ala Ala Arg Arg Gln Leu Lys Gln Met Glu Ser Gln Pro Leu Pro Gly Glu Arg
230 240
Ala Arg Ser Thr Leu Gln Lys Glu Val His Ala Ala Lys Ser Leu Ala Ile Ile Val Gly
250 260
Leu Phe Ala Leu Cys Trp Leu Pro Leu His Ile Ile Asn Cys Phe Thr Phe Phe Cys Pro
270 280
Asp Cys Ser His Ala Pro Leu Trp Leu Met Tyr Leu Ala Ile Val Leu Ser His Thr Asn
290 300
Ser Val Val Asn Pro Phe Ile Tyr Ala Tyr Arg Ile Arg Glu Phe Arg Gln Thr Phe Arg
310 320
Lys Ile Ile Arg Ser His Val Leu Arg Gln Gln Glu Pro Phe Lys Ala Ala Gly Thr Ser
330 340
Ala Arg Val Leu Ala Ala His Gly Ser Asp Gly Glu Gln Val Ser Leu Arg Leu Asn Gly
350 360
His Pro Pro Gly Val Trp Ala Asn Gly Ser Ala Pro His Pro Glu Arg Arg Pro Asn Gly
370 380
Tyr Ala Leu Gly Leu Val Ser Gly Gly Ser Ala Gln Glu Ser Gln Gly Asn Thr Gly Leu
390 400
Pro Asp Val Glu Leu Leu Ser His Glu Leu Lys Gly Val Cys Pro Glu Pro Pro Gly Leu
410
Asp Asp Pro Leu Ala Gln Asp Gly Ala Gly Val Ser

FIG. 3

```
        10                   30                   50
atgcccatca tgggctcctc ggtgtacatc acggtggagc tggccattgc tgtgctggcc
        70                   90                  110
atcctgggca atgtgctggt gtgctgggcc gtgtggctca acagcaacct gcagaacgtc
       130                  150                  170
accaactact ttgtggtgtc actggcggcg gccgacatcg cagtgggtgt gctcgccatc
       190                  210                  230
ccctttgcca tcaccatcag caccgggttc tgcgctgcct gccacggctg cctcttcatt
       250                  270                  290
gcctgcttcg tcctggtcct cacgcagagc tccatcttca gtctcctggc catcgccatt
       310                  330                  350
gaccgctaca ttgccatccg catcccgctc cggtacaatg gcttggtgac cggcacgagg
       370                  390                  410
gctaagggca tcattgccat ctgctgggtg ctgtcgtttg ccatcggcct gactcccatg
       430                  450                  470
ctaggttgga acaactgcgg tcagccaaag gagggcaaga accactccca gggctgcggg
       490                  510                  530
gagggccaag tggcctgtct ctttgaggat gtggtcccca tgaactacat ggtgtacttc
       550                  570                  590
aacttctttg cctgtgtgct ggtgcccctg ctgctcatgc tgggtgtcta tttgcggatc
       610                  630                  650
ttcctggcgg cgcgacgaca gctgaagcag atggagagcc agcctctgcc gggggagcgg
```

FIG. 4A

```
       670                       690                       710
gcacggtcca cactgcagaa ggaggtccat gctgccaagt cactggccat cattgtgggg
       730                       750                       770
ctctttgccc tctgctggct gcccctacac atcatcaact gcttcacttt cttctgcccc
       790                       810                       830
gactgcagcc acgcccctct ctggctcatg tacctggcca tcgtcctctc ccacaccaat
       850                       870                       890
tcggttgtga atcccttcat ctacgcctac cgtatccgcg agttccgcca gaccttccgc
       910                       930                       950
aagatcattc gcagccacgt cctgaggcag caagaacctt tcaaggcagc tggcaccagt
       970                       990                      1010
gcccgggtct tggcagctca tggcagtgac ggagagcagg tcagcctccg tctcaacggc
      1030                      1050                      1070
cacccgccag gagtgtgggc caacggcagt gctccccacc ctgagcggag gcccaatggc
      1090                      1110                      1130
tatgccctgg ggctggtgag tggagggagt gcccaagagt cccaggggaa cacgggcctc
      1150                      1170                      1190
ccagacgtgg agctccttag ccatgagctc aagggagtgt gcccagagcc ccctggccta
      1210                      1230
gatgaccccc tggcccagga tggagcagga gtgtcctga
```

FIG. 4B

10 20
Met Leu Leu Glu Thr Gln Asp Ala Leu Tyr Val Ala Leu Glu Leu Val Ile Ala Ala Leu

30 40
Ser Val Ala Gly Asn Val Leu Val Cys Ala Ala Val Gly Thr Ala Asn Thr Leu Gln Thr

50 60
Pro Thr Asn Tyr Phe Leu Val Ser Leu Ala Ala Ala Asp Val Ala Val Gly Leu Phe Ala

70 80
Ile Pro Phe Ala Ile Thr Ile Ser Leu Gly Phe Cys Thr Asp Phe Tyr Gly Cys Leu Phe

90 100
Leu Ala Cys Phe Val Leu Val Leu Thr Gln Ser Ser Ile Phe Ser Leu Leu Ala Val Ala

110 120
Val Asp Arg Tyr Leu Ala Ile Cys Val Pro Leu Arg Tyr Lys Ser Leu Val Thr Gly Thr

130 140
Arg Ala Arg Gly Val Ile Ala Val Leu Trp Val Leu Ala Phe Gly Ile Gly Leu Thr Pro

150 160
Phe Leu Gly Trp Asn Ser Lys Asp Ser Ala Thr Asn Asn Cys Thr Glu Pro Trp Asp Gly

170 180
Thr Thr Asn Glu Ser Cys Cys Leu Val Lys Cys Leu Phe Glu Asn Val Val Pro Met Ser

190 200
Tyr Met Val Tyr Phe Asn Phe Phe Gly Cys Val Leu Pro Pro Leu Leu Ile Met Leu Val

210 220
Ile Tyr Ile Lys Ile Phe Leu Val Ala Cys Arg Gln Leu Gln Arg The Glu Leu Met Asp

230 240
His Ser Arg Thr Thr Leu Gln Arg Glu Ile His Ala Ala Lys Ser Leu Ala Met Ile Val

250 260
Gly Ile Phe Ala Leu Cys Trp Leu Pro Val His Ala Val Asn Cys Val Thr Leu Phe Gln

270 280
Pro Ala Gln Gly Lys Asn Lys Pro Lys Trp Ala Met Asn Met Ala Ile Leu Leu Ser His

290 300
Ala Asn Ser Val Val Asn Pro Ile Val Tyr Ala Tyr Arg Asn Arg Asp Phe Arg Tyr Thr

310 320
Phe His Lys Ile Ile Ser Arg Tyr Leu Leu Cys Gln Ala Asp Val Lys Ser Gly Asn Gly

330
Gln Ala Gly Val Gln Pro Ala Leu Gly Val Gly Leu

FIG. 5

```
        10                            30                            50
atgctgctgg agacacagga cgcgctgtac gtggcgctgg agctggtcat cgccgcgctt
        70                            90                           110
tcggtggcgg gcaacgtgct ggtgtgcgcc gcggtgggca cggcgaacac tctgcagacg
       130                           150                           170
cccaccaact acttcctggt gtccctggct gcggccgacg tggccgtggg gctcttcgcc
       190                           210                           230
atccccttttg ccatcaccat cagcctgggc ttctgcactg acttctacgg ctgcctcttc
       250                           270                           290
ctcgcctgct tcgtgctggt gctcacgcag agctccatct tcagccttct ggccgtggca
       310                           330                           350
gtcgacagat acctggccat ctgtgtcccg ctcaggtata aaagtttggt cacggggacc
       370                           390                           410
cgagcaagag ggtcattgc tgtcctctgg gtccttgcct ttggcatcgg attgactcca
       430                           450                           470
ttcctggggt ggaacagtaa agacagtgcc accaacaact gcacagaacc ctgggatgga
       490                           510                           530
accacgaatg aaagctgctg ccttgtgaag tgtctctttg agaatgtggt ccccatgagc
       550                           570                           590
tacatggtat atttcaattt ctttgggtgt gttctgcccc cactgcttat aatgctggtg
       610                           630                           650
atctacatta agatcttcct ggtggcctgc aggcagcttc agcgcactga gctgatggac
```

FIG. 6A

```
        670                 690                 710
cactcgagga ccaccctcca gcgggagatc catgcagcca agtcactggc catgattgtg
        730                 750                 770
gggattttg ccctgtgctg gttacctgtg catgctgtta actgtgtcac tcttttccag
        790                 810                 830
ccagctcagg gtaaaaataa gcccaagtgg gcaatgaata tggccattct tctgtcacat
        850                 870                 890
gccaattcag ttgtcaatcc cattgtctat gcttaccgga accgagactt ccgctacact
        910                 930                 950
tttcacaaaa ttatctccag gtatcttctc tgccaagcag atgtcaagag tgggaatggt
        970                 990
caggctgggg tacagcctgc tctcggtgtg ggcctatga
```

FIG. 6B

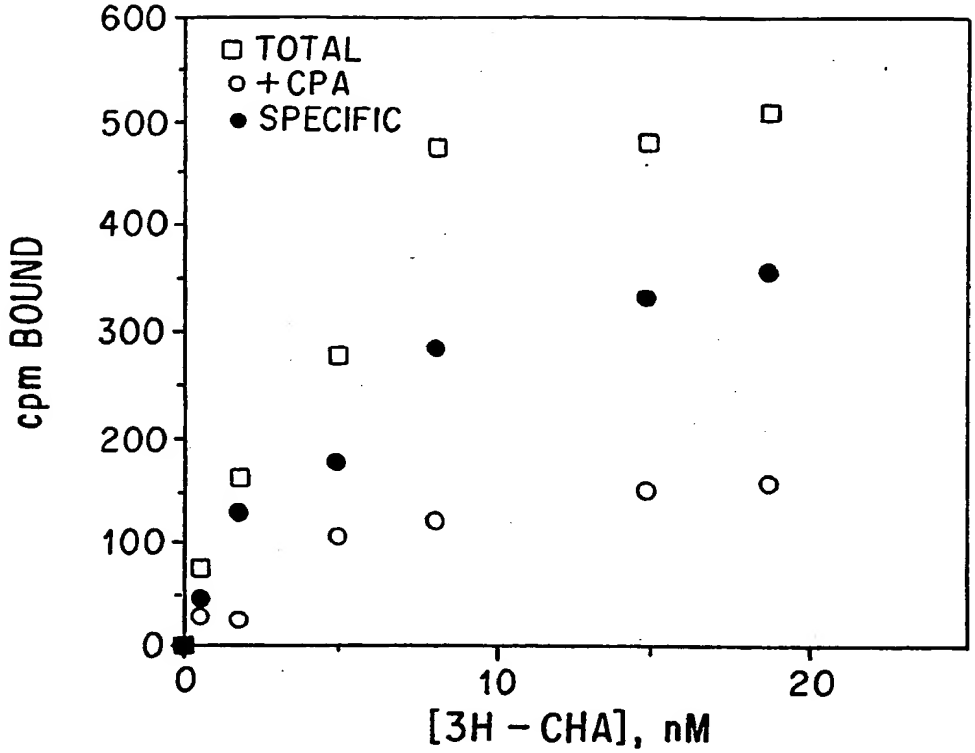
600
500
400
300
200
100
0
cpm BOUND
TOTAL
+CPA
SPECIFIC
0
10
20
[3H - CHA], nM

FIG. 7

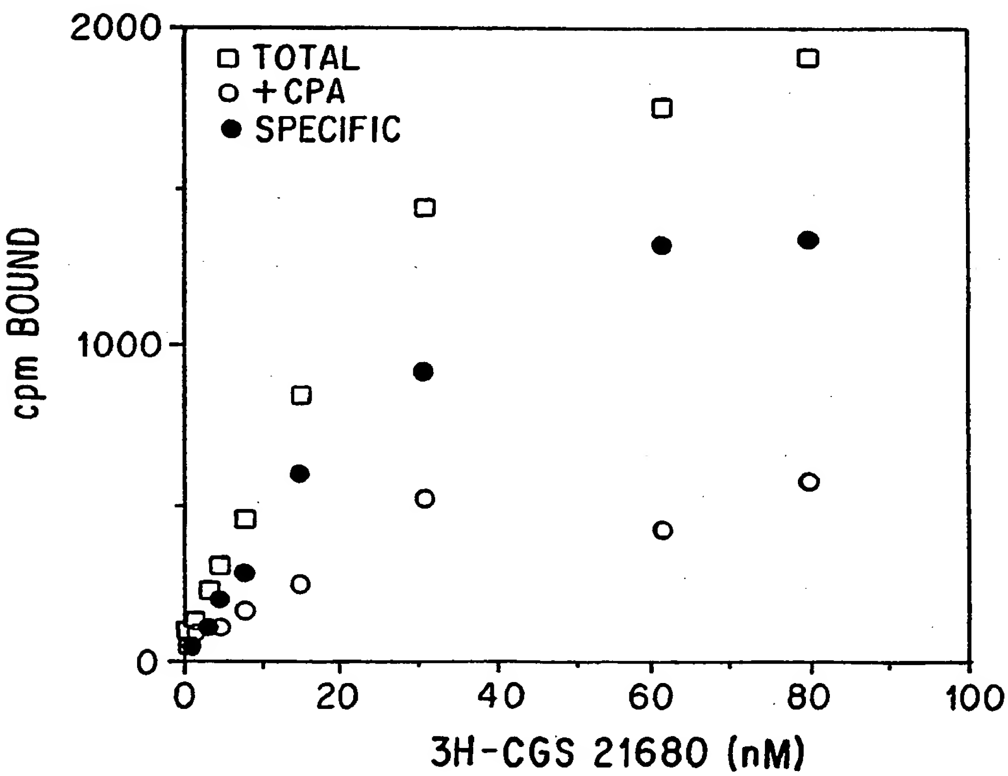
2000
1000
0
cpm BOUND
TOTAL
+CPA
SPECIFIC
0
20
40
60
80
100
3H-CGS 21680 (nM)

FIG. 8

10 20
Met Pro Asn Asn Ser Thr Ala Leu Ser Leu Ala Asn Val Thr Tyr Ile Thr Met Glu Ile
30 40
Phe Ile Gly Leu Cys Ala Ile Val Gly Asn Val Leu Val Ile Cys Val Val Lys Leu Asn
50 60
Pro Ser Leu Gln Thr Thr Thr Phe Tyr Phe Ile Val Ser Leu Ala Leu Ala Asp Ile Ala
70 80
Val Gly Val Leu Val Met Pro Leu Ala Ile Val Val Ser Leu Gly Ile Thr Ile His Phe
90 100
Tyr Ser Cys Leu Phe Met Thr Cys Leu Leu Leu Ile Phe Thr His Ala Ser Ile Met Ser
110 120
Leu Leu Ala Ile Ala Val Asp Arg Tyr Leu Arg Val Lys Leu Thr Val Arg Tyr Lys Arg
130 140
Val Thr Thr His Arg Arg Ile Trp Leu Ala Leu Gly Leu Cys Trp Leu Val Ser Phe Leu
150 160
Val Gly Leu Thr Pro Met Phe Gly Trp Asn Met Lys Leu Thr Ser Glu Tyr His Arg Asn
170 180
Val Thr Phe Leu Ser Cys Gln Phe Val Ser Val Met Arg Met Asp Tyr Met Val Tyr Phe
190 200
Ser Phe Leu Thr Trp Ile Phe Ile Pro Leu Val Val Met Cys Ala Ile Tyr Leu Asp Ile
210 220
Phe Tyr Ile Ile Arg Asn Lys Leu Ser Leu Asn Leu Ser Asn Ser Lys Glu Thr Gly Ala
230 240
Phe Tyr Gly Arg Glu Phe Lys Thr Ala Lys Ser Leu Phe Leu Val Leu Phe Leu Phe Ala
250 260
Leu Ser Trp Leu Pro Leu Ser Ile Ile Asn Cys Ile Ile Tyr Phe Asn Gly Glu Val Pro
270 280
Gln Leu Val Leu Tyr Met Gly Ile Leu Leu Ser His Ala Asn Ser Met Met Asn Pro Ile
290 300
Val Tyr Ala Tyr Lys Ile Lys Lys Phe Lys Glu Thr Tyr Leu Leu Ile Leu Lys Ala Cys
310
Val Val Cys His Pro Ser Asp Ser Leu Asp Thr Ser Ile Glu Lys Asn Ser Glu

FIG. 9

```
        10                    30                    50
atgcccaaca acagcactgc tctgtcattg gccaatgtta cctacatcac catggaaatt
        70                    90                   110
ttcattggac tctgcgccat agtgggcaac gtgctggtca tctgcgtggt caagctgaac
       130                   150                   170
cccagcctgc agaccaccac cttctatttc attgtctctc tagccctggc tgacattgct
       190                   210                   230
gttggggtgc tggtcatgcc tttggccatt gttgtcagcc tgggcatcac aatccacttc
       250                   270                   290
tacagctgcc tttttatgac ttgcctactg cttatcttta cccacgcctc catcatgtcc
       310                   330                   350
ttgctggcca tcgctgtgga ccgatacttg cgggtcaagc ttaccgtcag atacaagagg
       370                   390                   410
gtcaccactc acagaagaat atggctggcc ctgggccttt gctggctggt gtcattcctg
       430                   450                   470
gtgggattga cccccatgtt tggctggaac atgaaactga cctcagagta ccacagaaat
       490                   510                   530
gtcaccttcc tttcatgcca atttgtttcc gtcatgagaa tggactacat ggtatacttc
       550                   570                   590
agcttcctca cctggatttt catcccccctg gttgtcatgt gcgccatcta tcttgacatc
       610                   630                   650
ttttacatca ttcggaacaa actcagtctg aacttatcta actccaaaga gacaggtgca
```

FIG. 10A

```
       670                      690                      710
ttttatggac  gggagttcaa  gacggctaag  tccttgtttc  tggttctttt  cttgtttgct
       730                      750                      770
ctgtcatggc  tgcctttatc  tatcatcaac  tgcatcatct  actttaatgg  tgaggtacca
       790                      810                      830
cagcttgtgc  tgtacatggg  catcctgctg  tcccatgcca  actccatgat  gaaccctatc
       850                      870                      890
gtctatgcct  ataaaataaa  gaagttcaag  gaaacctacc  ttttgatcct  caaagcctgt
       910                      930                      950
gtggtctgcc  atccctctga  ttctttggac  acaagcattg  agaagaattc  tgagtag
```

FIG. 10B

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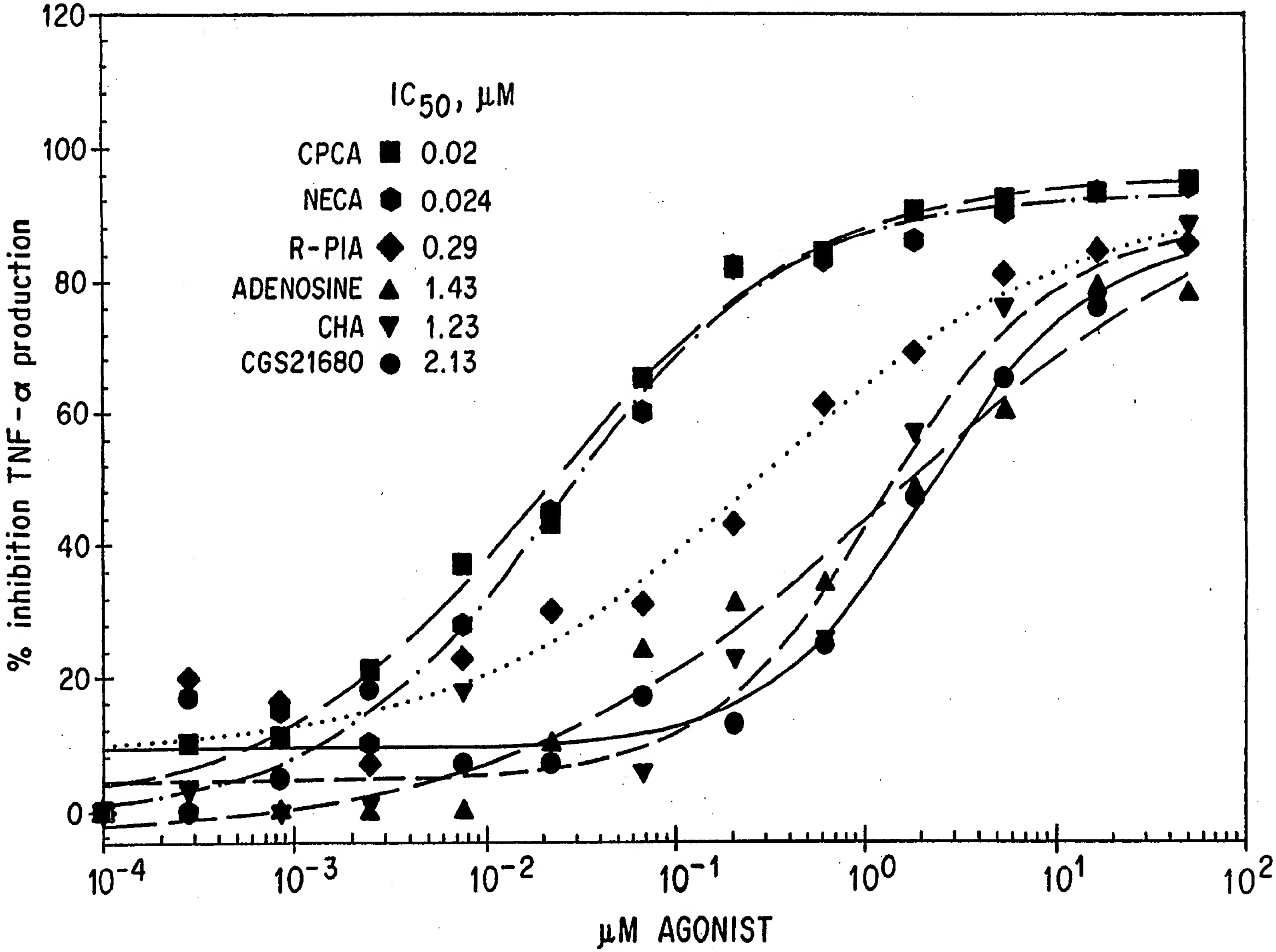


IC50, µM
CPCA 0.02
NECA 0.024
R-PIA 0.29
ADENOSINE 1.43
CHA 1.23
CGS21680 2.13
% inhibition TNF-α production
120
100
80
60
40
20
0
10^-4
10^-3
10^-2
10^-1
10^0
10^1
10^2
µM AGONIST

FIG. 11

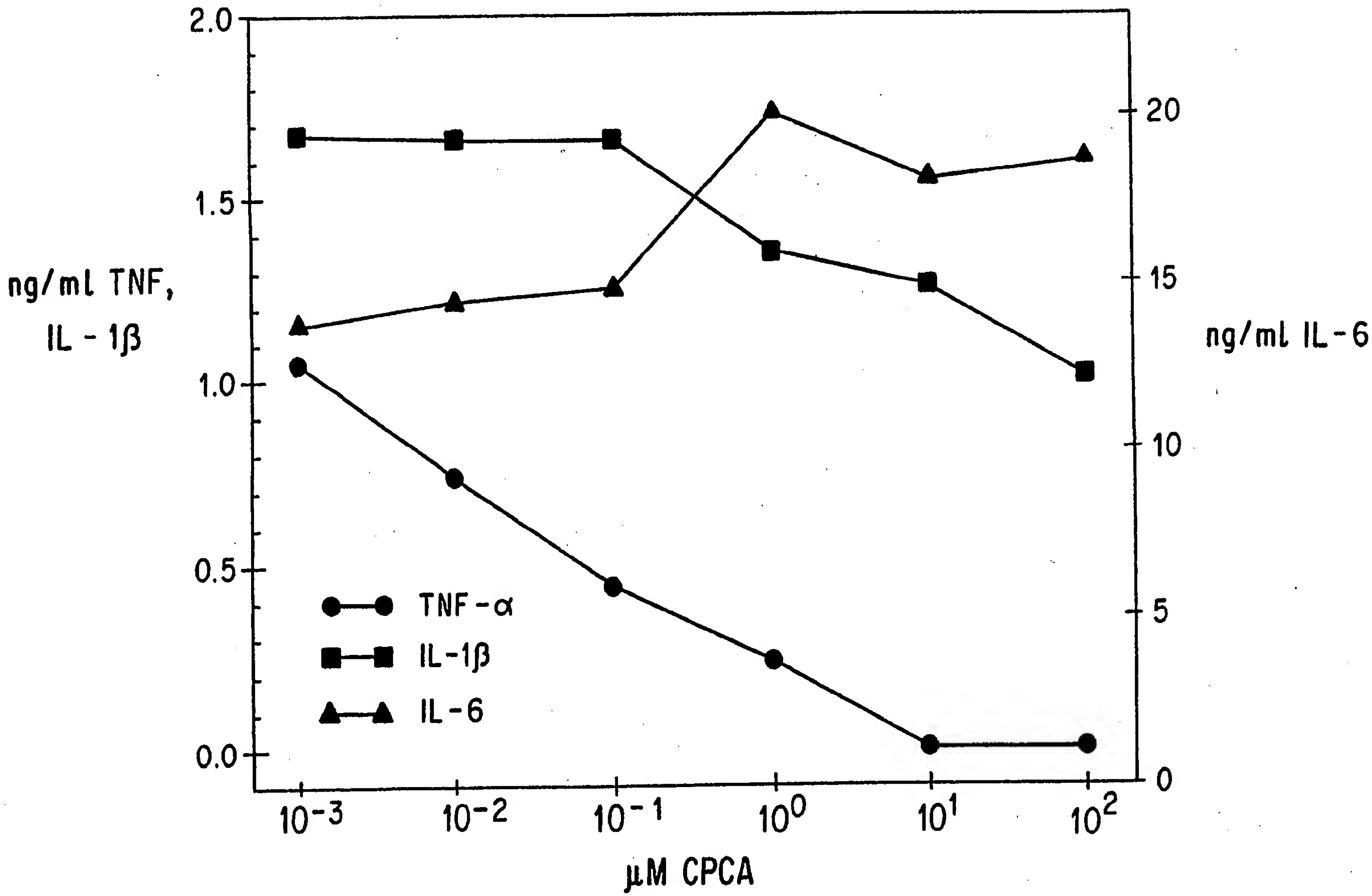

2.0
1.5
1.0
0.5
0.0
ng/ml TNF,
IL - 1β
20
15
10
5
0
ng/ml IL-6
TNF-α
IL-1β
IL-6
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
μM CPCA

FIG. 12

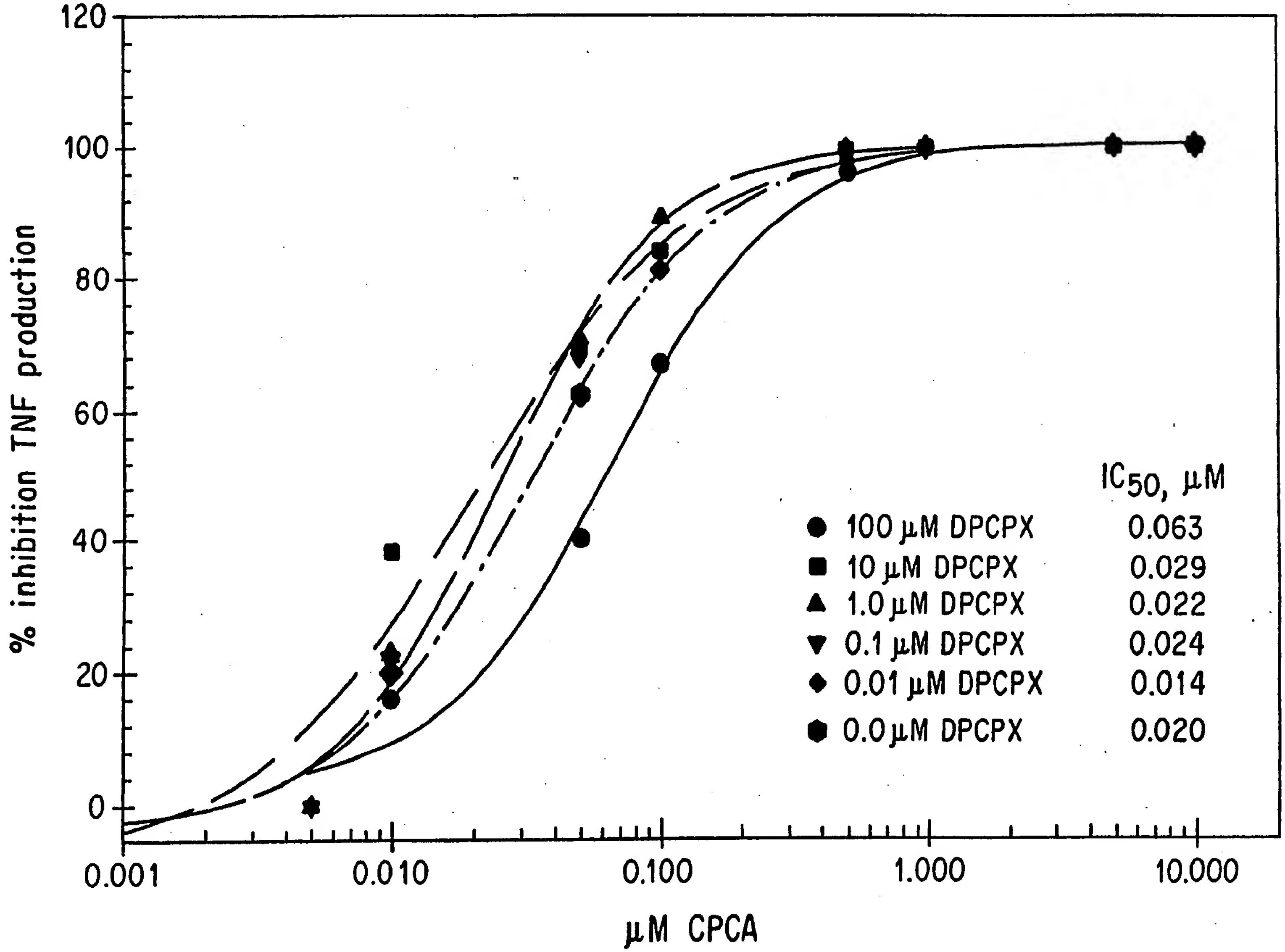
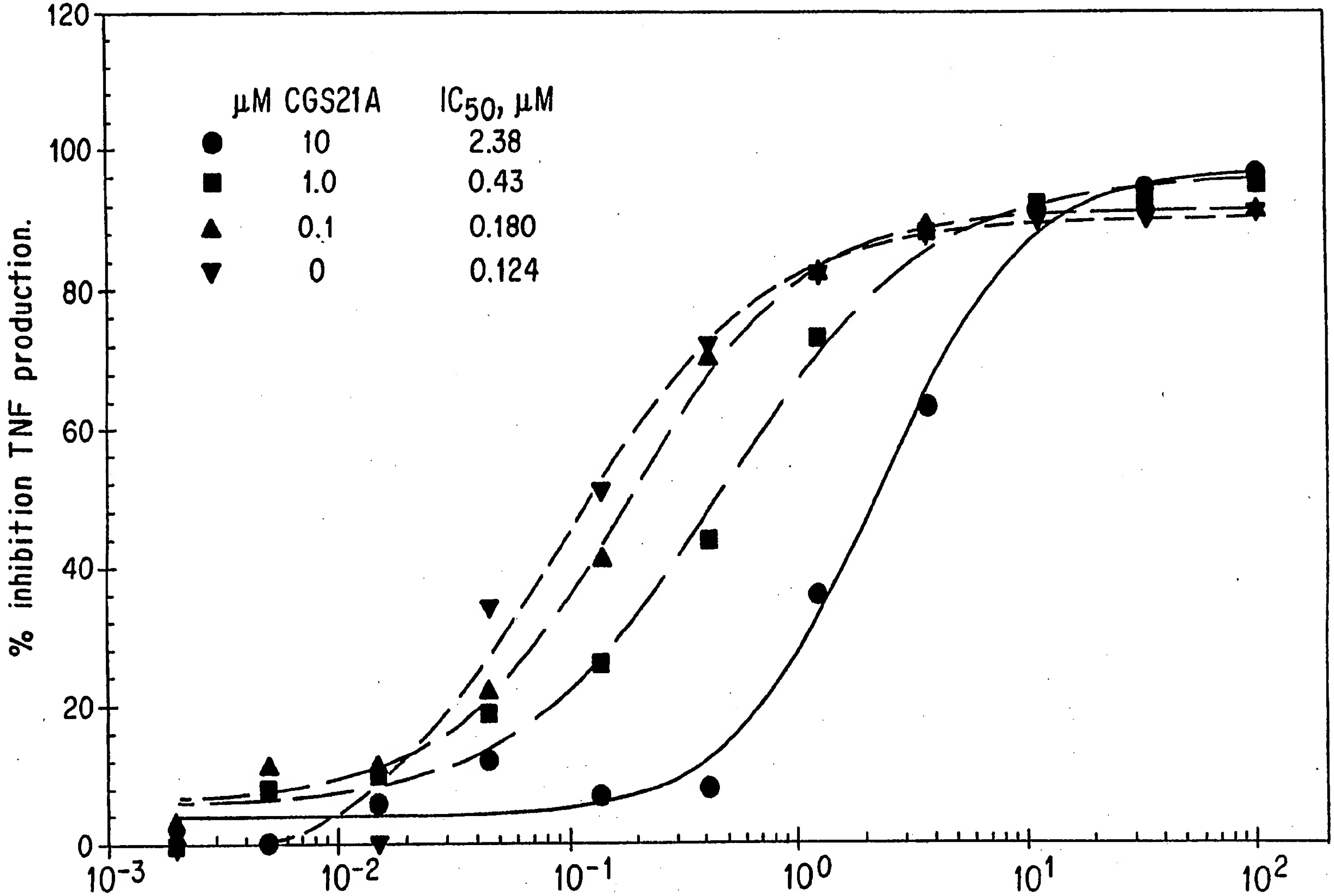

% inhibition TNF production
120
100
80
60
40
20
0
0.001
0.010
0.100
1.000
10.000
µM CPCA
IC50, µM
100 µM DPCPX 0.063
10 µM DPCPX 0.029
1.0 µM DPCPX 0.022
0.1 µM DPCPX 0.024
0.01 µM DPCPX 0.014
0.0 µM DPCPX 0.020

FIG. 13


µM CGS21A
IC50, µM
10
2.38
1.0
0.43
0.1
0.180
0
0.124
% inhibition TNF production.
120
100
80
60
40
20
0
10-3
10-2
10-1
10^0
10^1
10^2
µM CPCA

F I G. 14

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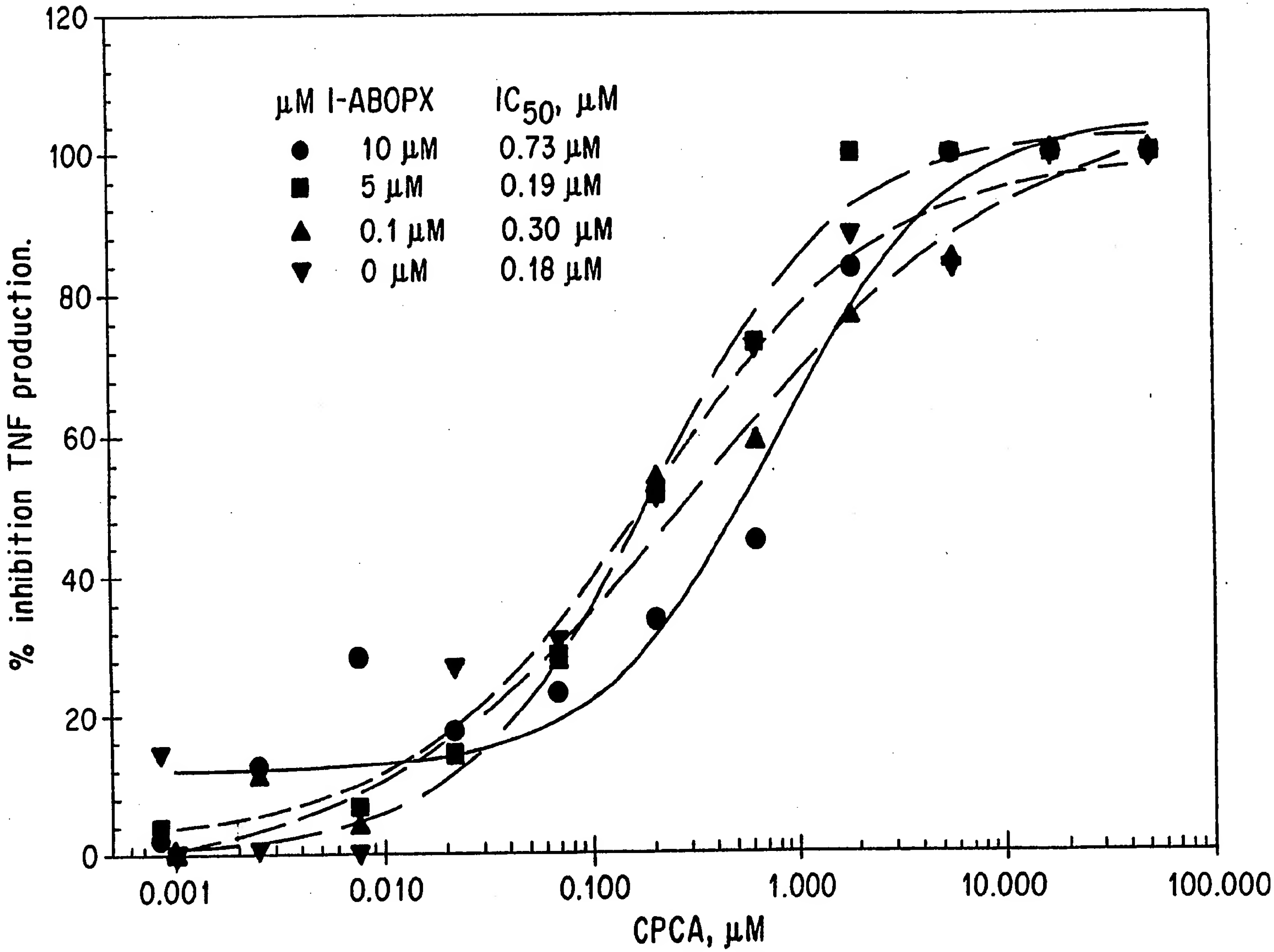

µM I-ABOPX
IC50, µM
10 µM
0.73 µM
5 µM
0.19 µM
0.1 µM
0.30 µM
0 µM
0.18 µM
% inhibition TNF production.
120
100
80
60
40
20
0
0.001
0.010
0.100
1.000
10.000
100.000
CPCA, µM

F I G. 15

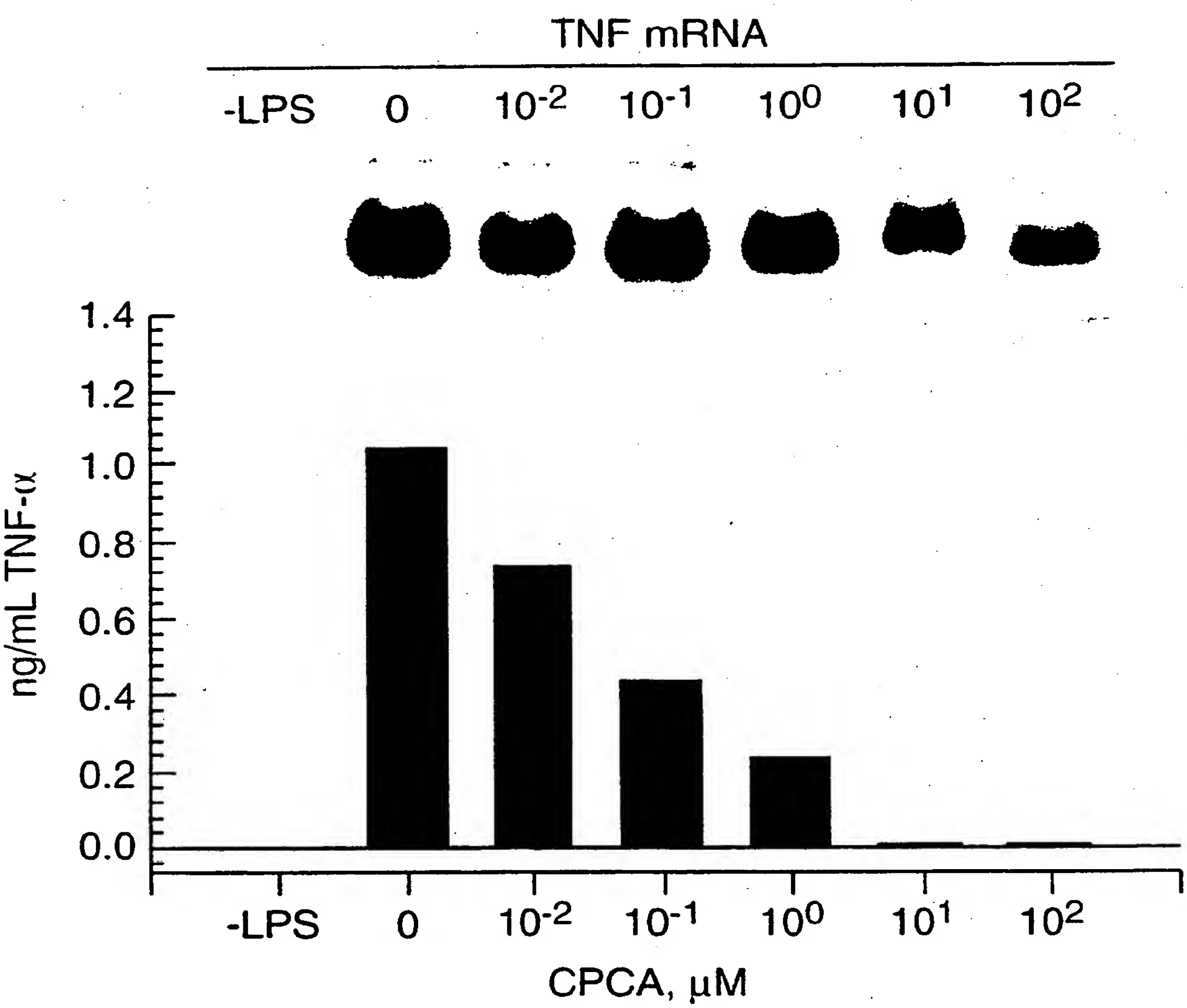
TNF mRNA
-LPS
0
10-2
10-1
100
101
102
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ng/mL TNF-α
-LPS
0
10-2
10-1
100
101
102
CPCA, μM

FIG.16

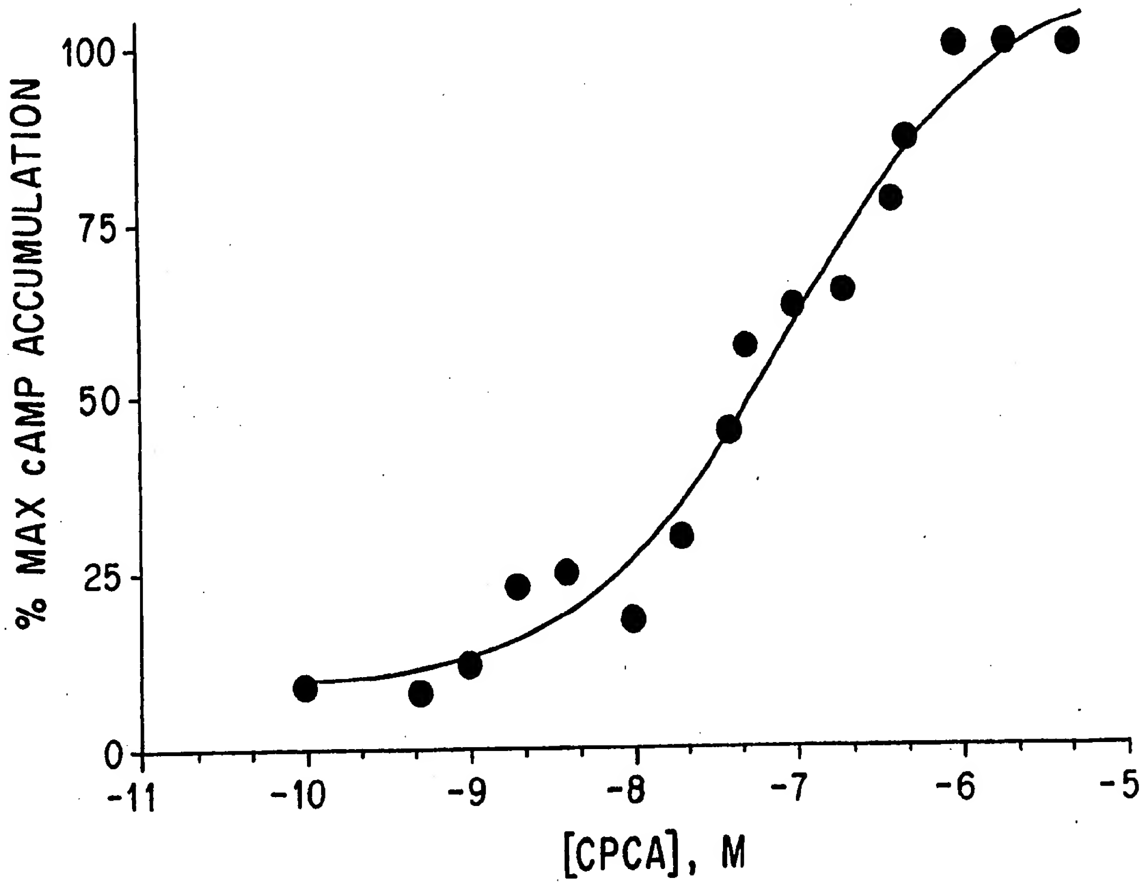

FIG. 17

2289218

TITLE OF THE INVENTION

INHIBITION OF TNFα PRODUCTION BY A2b ADENOSINE RECEPTOR AGONISTS AND ENHANCERS

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION:

The present invention concerns the use of compounds identified as specific modulators of adenosine's physiological actions. The pharmacology of these compounds is characterized through the use of cloned human adenosine A1, A2a, A2b and A3 receptor subtypes. This invention discloses that compounds identified as agonists of the A2b adenosine receptor subtype are useful in inhibiting the production of tumor necrosis factor (TNFα) by monocytes and/or macrophages. Therefore this invention comprises a method of treatment or prevention of disease states induced by production of TNFα. These conditions include, but are not limited to autoimmune diseases including rheumatoid arthritis, rheumatoid spondylitis, inflammatory bowl disease (ulcerative colitis and Crohns disease), intestinal pathology associated with graft vs. host disease, organ transplant reactions, septic shock, fever and myalgia due to infection and cachexia associated with chronic infections, malignancy and aquired immune deficiency syndrome, pulmonary diseases such as pulmonary sarcoidosis, silicosis, chronic pulmonary inflammatory disease, adult respiratory distress syndrome.

2. BACKGROUND:

Adenosine is a naturally occurring nucleoside which exhibits diverse and potent physiological actions in the cardiovascular, nervous, pulmonary, renal and immune systems. Adenosine has been demonstrated to terminate superventricular tachycardia through blockage of atrioventricular nodal conduction (J.P. DiMarco, et al., (1985) J. Am. Col. Cardiol. 6:417-425, A. Munoz, et al., (1984) Eur. Heart J. 5:735-738). Adenosine is a potent vasodilator except in the kidney and placenta (R.A. Olsson, (1981) Ann. Rev. Physiol. 43:385-

395). Adenosine produces bronchoconstriction in asthmatics but not in nonasthmatics (Cushly et al., 1984, Am. Rev. Respir. Dis. 129:380-384). Adenosine has been implicated as a preventative agent and in treatment of ventricular dysfunction following episodes of regional or global ischemia (M.B. Forman and C.E. Velasco (1991) Cardiovasc. Drugs and Therapy 5:901-908) and in cerebral ischemia(M.C. Evans, et al., (1987) Neurosci. Lett. 83:287, D.K.J.E.,Von Lubitz, et al., (1988) Stroke 19:1133).

Dog A1 and A2a adenosine receptors were the first adenosine receptors to be cloned. See F. Libert, et al., (1989) Science 244:569-572,. C. Maennant, et al., Biochem. Biophys. Res. Comm., (1990) 173:1169-1178, and F. Libert, et al. (1991) EMBO J. 10:1677-1682. The rat A1 adenosine receptor was cloned by L.C. Mahan, et al., (1991) Mol. Pharm. 40:1-7 and S.M. Reppert, et al., (1991) Mol. Endocrin. 5:1037-1048, the rat A2a by Fink et. al., (1992) Mol. Brain Res. 14:186-195, and the rat A2b by Stehle et al. (1992) Mol. Endocrinol. 6:384-393. Cloning of the rat A3 adenosine receptor was reported by Meyerhof et al., (1991) FEBS Lett. 284:155-160 and Zhou et al., (1992) PNAS USA 89:7432-7436. Cloning of the sheep A3 adenosine receptor has been reported by Linden et al., (1993) Mol. Pharm. 44:524-532. Cloning of the human A1, A2a, A2b and A3 receptors were reported in GB 2264948-A (9/15/93). The human A1 adenosine receptor differs by 18 amino acids from the dog A1 sequence and 16 amino acids from the rat A1 sequence. The human A2a adenosine receptor differs by 28 and 71 amino acids, respectively from the dog and rat A2a sequences. The amino acid sequence for the human A3 receptor is 72% identical with the rat A3 receptor and 85% identical with the sheep A3 receptor sequences.

The actions of adenosine are mediated through G-protein coupled receptors, the A1, A2a, A2b and A3 adenosine receptors. The adenosine receptors were initially classified into A1 and A2 subtypes on the basis of pharmacological criteria and coupling to adenylate cyclase (Van Caulker, D., Muller, M. and Hamprecht, B. (1979) J. Neurochem. **33**, 999-1003.). Further pharmacological classification of adenosine

receptors prompted subdivision of the A2 class into A2a and A2b subtypes on the basis of high and low affinity, respectively, for adenosine and the agonists NECA and CGS-21680 (Bruns, R.F., Lu, G.H. and Pugsley, T.A. (1986) Mol. Pharmacol. **29**, 331-346; Wan, W., Sutherland, G.R. and Geiger, J.D. (1990) J. Neurochem. **55**, 1763-1771). The existence of A1, A2a and A2b subtypes has been confirmed by cloning and functional characterization of expressed bovine, canine, rat and human receptors. A fourth subtype, A3, had remained pharmacologically undetected until its recent identification by molecular cloning. The rat A3 sequence, tgpcr1, was first cloned from rat testis by Meyerhoff et al. (see above). Subsequently, a cDNA encoding the identical receptor was cloned from striatum and functionally expressed by Zhou et al. (see above). When compared to the other members of the G-protein coupled receptor family, the rat sequence had the highest homology with the adenosine receptors (> 40% overall identity, 58% within the transmembrane regions). When stably expressed in CHO cells, the receptor was found to bind the radioligand ^{125}I-APNEA (N^6-2-(4-amino-3-iodophenyl)ethyladenosine) and when transfected cells were treated with adenosine agonists, cyclic AMP accumulation was inhibited with a potency order of NECA = R-PIA > CGS21680. The rat A3 receptor exhibited a unique pharmacology relative to the A1 and A2 adenosine receptor subtypes and was reported not to bind the xanthine antagonists 1,3-dipropyl-8-phenylxanthine (DPCPX) and xanthine amine congener (XAC). Messenger RNA for the rat A3 adenosine receptor is primarily expressed in the testis.

The sheep homolog of the A3 receptor was cloned from hypophysial pars tuberalis (see Linden et al. above). The sheep receptor is 72% identical to the rat receptor, binds the radioligand ^{125}I-ABA and is also coupled to inhibition of cyclic AMP. The agonist affinity order of the sheep receptor is I-ABA > APNEA> NECA $\geq$ R-PIA >> CPA. The pharmacology of xanthine antagonists was extensively studied and the sheep receptor was found to exhibit high affinity for 8-phenylxanthines with para-acidic substitutions. In contrast to the rat transcript, the expression of the sheep A3 adenosine receptor transcript

is widespread throughout the brain and is most abundant in the lung and spleen. Moderate amounts of transcript are also observed in pineal and testis. The cloning and pharmacological profile of the human A3 adenosine receptor was disclosed by Salvatore et al., [P.N.A.S. 90:10365-10369, 1993] and is quite similar to that of the sheep A3 receptor pharmacology.

Based on the use of these cloned receptors, an assay has been described to identify adenosine receptor agonists and antagonists and determine their binding affinity (see GB 2 264 948 A, published 9/15/93; see also R.F. Bruns, et al., (1983) Proc. Natl. Acad. Sci. USA 80:2077-2080; R.F. Bruns, et al.,(1986) Mol. Pharmacol. 29:331-346; M.F. Jarvis, et al. (1989) J. Pharma. Exp. Therap. 251:888-893; K.A. Jacobson et al., (1989) J. Med. Chem. 32:1043-1051).

Adenosine receptor agonists, antagonists and binding enhancers have been identified and implicated for usage in the treatment of physiological complications resulting from cardiovascular, pulmonary, renal and neurological disorders. Adenosine receptor agonists have been identified for use as vasodilators ((1989) FASEB. J. 3(4) Abs 4770 and 4773, (19910 J. Med. Chem. (1988) 34:2570), antihypertensive agents (D.G. Taylor et al., FASEB J. (1988) 2:1799), and anti-psychotic agents (T.G. Heffner et al., (1989) Psychopharmacology 98:31-38). Adenosine receptor agonists have been identified for use in improving renal function (R.D. Murray and P.C. Churchill,(1985) J. Pharmacol. Exp. Therap. 232:189-193). Adenosine receptor allosteric or binding enhancers have shown utility in the treatment of ischemia, seizures or hypoxia of the brain (R.F. Bruns, et al. (1990) Mol. Pharmacol. 38:939-949; C.A. Janusz, et al., (1991) Brain Research 567:181-187). The cardioprotective agent, 5-amino-4-imidazole carboxamide (AICA) ribose has utility in the treatment of ischemic heart conditions, including unstable angina and acute myocardial infarction (H.E. Gruber, et al. (1989) Circulation 80: 1400-1414).

Through the use of homogeonous, recombinant adenosine receptors, the identification and evaluation of compounds which have

selectivity for a single receptor subtype is now possible. Because of the variable effects of adenosine documented in other species, the utilization of human adenosine receptor subtypes is advantageous for the development of human therapeutic adenosine receptor agonists, antagonists or enhancers.

The anti-inflammatory properties of adenosine have been documented. Adenosine receptor agonists inhibit TNFα production by LPS-stimulated human monocytes (Vraux, et al. 1993 Life Sci. 52:1917-1924) with an affinity profile which does not correspond to A1 or A2a subtype pharmacology. The identification of the specific adenosine receptor subtype mediating the inhibition of TNFα has not been elucidated. With the use of affinity order profiles generated with adenosine receptor agonists, subtype selective adenosine receptor antagonists and information derived from the pharmacological characterization of the human A2b receptor cDNA stably expressed in CHO cells, I have identified the A2b adenosine receptor subtype in mediating the inhbition of TNFα in stimulated human monocytes.

The use of an A2b adenosine receptor specific agonist is advantageous over existing therapeutic agents in that a decrease or elimination of side effects experienced when non-selective agonists or the natural agonist, adenosine, are used for therapy. Allosteric effectors or enhancers of the A2b adenosine receptor would eliminate or decrease systemic side effects. Enhancers increase the binding of the native agonists and have been described for A1 adenosine receptors. A2b receptor enhancers remain pharmacologically silent in the absence of adenosine and act locally at sites of inflammation where increases in adenosine concentrations are realized, thereby reducing side effects. The use of such enhancers to inhibit TNFα production naturally forms part of the instant invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Full length amino acid sequence of human A1 adenosine receptor.

Figure 2 Full length nucleotide sequence of the cloned human A1 adenosine receptor complementary DNA depicted from the 5' to 3' terminus.

Figure 3 Full length amino acid sequence of human A2a adenosine receptor.

Figure 4 Full length nucleotide sequence of cloned human A2a adenosine receptor complementary DNA depicted from the 5' to 3' terminus.

Figure 5 Full length amino acid sequence of human A2b receptor.

Figure 6 Full length nucleotide sequence of cloned human A2b adenosine receptor complementary DNA depicted from the 5' to 3' terminus.

Figure 7 Saturation binding of [^{3}H]-cyclohexyladenosine (CHA) to human A1 adenosine receptor in COS7 assay.

Figure 8 Saturation binding of [^{3}H]-CGS21680 to human A2a adenosine receptor in COS7 assay.

Figure 9 Full length amino acid sequence of human A3 adenosine receptor.

Figure 10 Full length nucleotide sequence of the cloned human A3 adenosine receptor complementary DNA depicted from the 5' to 3' terminus.

Figure 11 Adenosine agonists inhibit LPS induced TNFα production in human blood monocytes with a rank order potency of CPCA $\geq$ NECA >> R-PIA > CHA $\geq$ adenosine > CGS21680. Human peripheral blood mononuclear cells

were cultured on plastic plates coated with fibronectin. The cells were treated with 100 ng/mL of LPS and the indicated concentrations of adenosine agonist. The TNFα levels were measured in cell-culture supernatant by specific ELISA after 18 hours of culture.

Figure 12 The adenosine agonist CPCA inhibits TNFα, but not IL1ß or IL-6 release from LPS stimulated human monocytes. Human peripheral blood monocytes were adhered to fibronectin coated plates and stimulated with LPS in the presence of the indicated concentrations of CPCA. Cell culture supernatant was removed after overnight incubation and tested by specific ELISA for IL-6, IL1ß, and TNFα. CPCA did not inhibit IL-6 or IL1ß production.

Figure 13 The A1 adenosine receptor antagonist DPCPX does not affect the CPCA induced inhibition of TNFα production in LPS stimulated monocytes. Human peripheral blood monocytes were adhered to fibronectin coated plates. The cells were treated with 100 ng/mL of LPS, and the indicated concentrations of CPCA and DPCPX. TNFα production levels were measured by specific ELISA in cell-culture supernatant after 18 hours of culture.

Figure 14 CG21A partially antagonizes CPCA induced inhibition of TNFα production in LPS stimulated monocytes. Human peripheral blood monocytes were adhered to fibronectin coated plates. The cells were treated with 100 ng/mL of LPS, and the indicated concentrations of CPCA and CG21A, an adenosine A2a receptor antagonist. TNFa production levels were measured by specific ELISA in cell-culture supernatant after 18 hours of culture. CGS21A inhibited TNFα production in a dose dependent manner in the absence of CPCA, consistent with the hypothesis that

endogenous adenosine partially represses TNFα production in the assay.

Figure 15 The A3 adenosine receptor antagonist I-ABOPX does not affect the CPCA induced inhibition of TNFα production in LPS stimulated monocytes. Human peripheral blood monocytes were adhered to fibronectin coated plates. The cells were treated with 100 ng/mL of LPS, and the indicated concentrations of CPCA and I-ABOPX. TNFα production levels were measured by specific ELISA in cell-culture supernatant after 18 hours of culture.

Figure 16 Northern blot analysis of the TNFα mRNA production in LPS stimulated monocytes treated with the adenosine agonist CPCA. Total RNA was extracted from 1 x 10^7 adhered human monocytes one hour following stimulation with LPS in the presence of the indicated concentrations of CPCA. Total RNA (10 μg) was blotted using a ^{32}P labeled cDNA probe. No significant reductions in TNFα mRNA production were observed using CPCA at levels sufficient to suppress protein production by greater than ten fold.

Figure 17 CPCA dose response of cAMP accumulation in CHO cells stably expressing the human A2b receptor.

SUMMARY OF THE INVENTION

Adenosine receptor agonists have been shown to inhibit tumor necrosis factor alpha (TNFα) production in lipopolysaccharide (LPS) stimulated monocytes with an affinity order profile of CPCA $\geq$ NECA >> R-PIA > CHA $\geq$ adenosine > CGS21680. This agonist profile does not correlate with either the A1 or A2a adenosine receptor subtype pharmacology. In order to define the receptor subtype mediating the inhibitory effect, adenosine receptor antagonists were

evaluated for their ability to block the inhibition of TNFα production caused by CPCA in LPS-stimulated human monocytes. The involvement of the A1 and A2a adenosine receptor subtypes was ruled out on the basis of the inability of DPCPX and 3-succinylamino-strylcaffine, CG21A, respectively, to appreciably antagonize the inhibition produced by CPCA. The A3 adenosine receptor specific antagonist IABOPX was also ineffective in blocking agonist induced inhibition of TNFα production. The agonist affinity order profile established for the monocyte adenosine receptor was similar to the A2b receptor in VA13 human fibroblasts and human erythroleukemic cells (HEL) defined by EC_{50} values for intracellular cyclic adenosine monophosphate (cAMP) accumulation. However, the potency of the agonists to inhibit TNFα production in monocytes was greater than values determined by increases in cAMP accumulation in fibroblasts or HEL cells. I have found that in stable CHO cells expresing the cloned human A2b cDNA, the potency (EC_{50}) of CPCA to induce cAMP accumulation was similar to the value obtained for inhibition of TNFα production in LPS-stimulated human monocytes. To define which adenosine receptor subtypes are present on monocytes, A1, A2a, A2b, and A3 adenosine receptor transcripts were detected by reverse transcriptase PCR (RT-PCR) of mRNA prepared from both LPS-stimulated and non-stimulated monocytes. The regulation of TNFα expression resulting from mediation at the A2b receptors is demonstrated to be consistent with a mechanism involving increased intracellular cAMP levels.

ABBREVIATIONS

[^{3}H]-CHA, [^{3}H]-cyclohexyladenosine; [^{3}H]-NECA, [^{3}H]-5'-N-ethyl-carboxamido-adenosine; ^{125}I-ABA, N^6-(4-amino-3-^{125}iodobenzyl)adenosine; ^{125}I-APNEA, N^6-2-(4-amino-3-^{125}iodophenyl)ethyladenosine; NECA, 5'-N-ethylcarboxamidoadenosine; CGS21680, 2-[4-(2-carboxyethyl)phenyl]ethylamino-5'-N-ethylcarboxamidoadenosine; (R,S)-PIA, (R,S)-N^6-phenyl-2-propyladenosine; CPA, N^6-cyclopentyladenosine; CPCA, 5'-(N-cyclopropyl)-carboxamidoadenosine; CG21A, 3-succinylaminostrylcaffine; I-ABOPX, (3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate)phenyl-1-prpopylxanthine; BW-A1433, 1,3-dipropyl-8-(4-acrylate)phenylxanthine; XAC, xanthine amine cogener; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; GTPγS, guanosine 5'-O-3-thiotriphosphate; Gpp(NH)p, 5'-guanylimidodiphosphate; G protein, guanine nucleotide-binding proteins.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a method for achieving specific inhibition of TNFα production through agonist stimulation of the A2b adenosine receptor. TNFα is a pro-inflammatory cytokine which, among other effects, induces fever and stimulates phospholipase A2 production. Lipopolysaccharide (LPS) is a biological mediator which gives rise to a number of adverse responses. A principal mediator to these effects is TNFα. A variety of adenosine receptor agonists have been tested for their ability to block LPS-mediated TNFα production in human monocytes [Le Vraux et al., Life Sciences 52:1917-1924, 1993]. Figure 11 summarizes the pharmacological profile of this effect [CPCA ≥ NECA >> R-PIA > CHA ≥ adenosine > CGS21680]. The conclusion reported in Le Vraux et al., based on this pharmacology, was that the inhibition of of TNFα production was probably mediated through the A3 adenosine receptor, or through an uncharacterized receptor, but not through the A1 or A2 adenosine receptors. As can be seen from this data, CPCA and NECA are the most potent inhibitors of TNFα

production. Both compounds have been characterized as binding both the A1 and the A2 adenosine receptor subtypes with high affinity, see the table below:

AFFINITY OF ADENOSINE ANALOGS FOR HUMAN ADENOSINE RECEPTOR SUBTYPES, Ki or Kd, µM *

Agonists	A1	A2a	A2b	A3
NECA	0.025	0.029	0.9 (a)	0.026
CPCA	0.006 (rat)	0.0134 (rat)	0.050 (a)	1.0
CGS21680	56	0.017	1600 (b)	5.6
R-PIA	0.003	0.127	160 (b)	0.034
CHA	0.002	0.6	280 (b)	n.d.
Antagonists				
DPCPX	0.0007	0.10	0.55 (b)	0.75
CGS21A	35 (rat)	0.143 (rat)	n.d.	>50

*Values determined in rat are indicated, otherwise all other data is from human, (a) EC50 values for cAMP accumulation in stable CHO cells expressing the human A2b cDNA; (b) EC50 values for cAMP accumulation in human erythroleukemic cells, HEL cells.

The A1 adenosine receptor selective agonists R-PIA and CHA are significantly less potent than CPCA or NECA. The A2a specific agonist CGS21680 was found to be the least potent of all. The rank order of potency of the compounds to inhibit TNFα production is not like that of either A1 or A2 [Le Vraux et al., Life Sciences 52:1917-1924, 1993]. The affinity order profile reported by Le Vraux et al. is similar to the agonist profile reported by Castanon and Spevak [BBRC 198:626-631, 1994] for the induction of cyclic adenosine monophosphate (cAMP) accumulation in stable CHO cell lines expressing the cloned A2b adenosine receptor. However, Castanon and Spevak did not study the role of the A2b receptor in inhibition of TNFα production. In addition, the agonist affinity order profile data reported by Le Vraux et al. for TNFα inhibition is not dissimilar from the agonist order profile reported by Salvatore et al., [P.N.A.S. 90:10365-10369, 1993] for the cloned A3 adenosine receptor and suggested that

the A3 receptor may be responsible for TNFα inhibition in LPS-stimulated monocytes. However, the potency of CPCA for the A3 receptor was not reported by Salvatore et al. and therefore, prior to this invention, the role of A3 adenosine receptor in the inhibition of TNFα production could not be ruled out and the specific adenosine receptor subtype which is responsible for inhibition of TNFα production could not be positively identified. This patent disclosure demonstrates that CPCA has a much lower affinity for the A3 receptor than it does for the A2b receptor and by using A3 adenosine receptor specific antagonists, the involvement of A3 receptor activation in the inhibition of TNFα production is definitively ruled out. This patent disclosure demonstrates that A1 and A2a adenosine receptors are not involved in the inhibition of TNFα production. This invention reveals that activation only at the A2b adenosine receptor is responsible for the inhibition of TNFα production.

The role of cAMP elevations has been correlated with the inhibition of LPS induced TNFα production defined through the use of the phosphodiesterase inhibitor pentoxifyllin [Strieter, et al., (1988) Biochem. Biophys. Res. Commun. 155: 1230-1236]. The inhibition of TNFα production through activation at A2b adenosine receptors on stimulated monocytes is therefore consistent with a mechanism resulting from increases in intracellular cAMP. Therefore, this invention comprises a method for inhibiting TNFα production specifically through A2b receptor activation.

Since Le Vraux et al., suggested that the receptor responsible for inhibition of TNFα production was possibly the A3 adenosine receptor and not the A1 or A2 receptors, I initiated the following studies in order to elucidate which receptor is, in fact, responsible for inhibition of TNFα production.

I confirmed that the A1 and A2a receptor subtypes are not responsible for the inhibition of TNFα production by using the A1 and

A2a adenosine receptor selective antagonists DPCPX and CG21A respectively. These compounds do not appreciably alter the IC_{50} of CPCA in antagonist competition experiments except at very high concentrations (see Figures 13 and 14). This data confirms that the A1 and A2a adenosine receptor subtypes are not involved in the inhibition of TNFα production. I confirmed that the A3 receptor subtype was not responsible for the inhibition of TNFα production by using the A3 specific antagonist, I-ABOPX (Figure 15). I-ABOPX did not alter the IC_{50} of CPCA inhibition of TNFα production.

I further determined that the affinity of CPCA for the A3 adenosine receptor subtype is 1 μM and therefore, the A3 receptor cannot be responsible for the inhibition of TNFα production induced by CPCA which exhibits a much higher (20,000-fold) affinity for the A2b than the A3 adenosine receptor. I obtained the EC_{50} value for CPCA induced cAMP accumulation in stable CHO cell lines expressing the human A2b receptor and found that the EC_{50} value is the same as that obtained from the stimulated monocytes (Figure 17). I further confirmed that the effect is specific for TNFα because IL1ß and IL-6 production are unaffected by treatment with CPCA, (Figure 12).

Northern blot data of total RNA from LPS stimulated monocytes indicates that titration of CPCA reduces the levels of secreted TNFα protein in a dose dependent manner, Figure 16. This data indicates that adenosine agonists inhibit TNFα production primarily through post-transcriptional mechanisms. This observation is consistent with reports that TNFα mRNA contains 3'-untranslated sequences that mediate translational activation in response to specific inducing signals (e.g. LPS). Removal of these sequences has been shown to result in the inability of the mRNA to be translated. Therefore, it appears that adenosine blocks components of the LPS signal transduction pathway that are related to these 3'-untranslated elements of the TNFα gene.

To define which adenosine receptor subtypes are present on monocytes, A1, A2a, A2b, and A3 adenosine receptor transcripts were detected by reverse transcriptase PCR (RT-PCR) of mRNA prepared from both LPS-stimulated and non-stimulated monocytes. All four

adenosine receptor subtypes were detected in mRNA prepared from both normal and LPS-stimulated monocytes. Even though all of the identified adenosine receptor subtypes are present on monocytes, this invention reveals that only the A2b receptor affects TNFα production.

Therefore, one embodiment of this invention is a method for identifying A2b adenosine receptor selective compounds which comprises the steps of:

(a) contacting monocytes with a test compound and measuring the effect of the test compound on TNFα production;

(b) contacting a test compound, identified according to step (a) as inhibiting TNFα production by the monocytes, with membranes derived from a stable cell line individually expressing each of the A1, A2a, A2b, or A3 adenosine receptor or with the whole cell individually expressing each of the A1, A2a, A2b, or A3 adenosine receptor and measuring the binding affinity of the test compound for the receptor or the effect of the test compound on cAMP production in the stable cell line;

(c) selecting compounds which bind to the A2b adenosine receptor or which induces elevation in cAMP in the cell line expressing the A2b adenosine receptor and which do not bind to membranes or affect the cAMP level in the stable cell lines expressing the A1, A2a, or A3 adenosine receptor subtypes.

This invention likewise comprises the use of compounds identified according to this method which have A2b adenosine receptor enhancer or agonist activities for the inhibition of TNFα production. This invention further comprises a method for inhibiting TNFα production by contacting monocytes with inhibitorily effective amounts of compounds that act as A2b adenosine receptor agonists. An inhibitorily effective amount of an A2b adenosine receptor agonist is, for example, 0.1 ng to 10 mg/kg per day of CPCA, NECA or a compound exhibiting similarly potent or more potent A2b adenosine receptor agonist properties.

The following examples are provided to further define but not to limit the invention defined by the foregoing description and the claims which follow:

EXAMPLE 1

STEP A:

In the first step of obtaining the partial cDNAs encoding the human A1 and A2a adenosine receptors, total RNA was extracted by homogenizing 2.3g human ventricle in 20 ml 5M guanidine isothiocyanate, 0.1M sodium citrate, pH 6.3, 1mM EDTA, pH 7.0, 5% beta-mercaptoethanol, and 0.5% sodium lauryl sarcosinate. The homogenate was centrifuged for 10 min. at 10,000 rpm and the resulting supernatant was layered onto a cushion of 5.7M CsCl/0.1M EDTA, pH 7.0. After 20 hrs. of centrifugation at 24,000 rpm, the resulting pellet was precipitated one time and then passed over an oligo(dT)-cellulose (PHARMACIA, Piscataway, NJ) column to isolate poly(A)+ RNA.

An oligo(dT) primed library was synthesized from 5 µg of the poly$(A)^+$ human ventricle RNA using the YOU-PRIME cDNA SYNTHESIS KIT (PHARMACIA, Piscataway, NJ). See Gubler and Hoffman Gene 25:263 (1983). The resulting double-stranded cDNA was ligated into λgt10 EcoRI arms (PROMEGA, Madison, WI) and packaged according to the GIGAPACK II GOLD PACKAGING EXTRACT protocol (STRATAGENE, La Jolla, CA). See Huynh et al. (1985) DNA Cloning Techniques: A Practical Approach, IRL Press, Oxford, p.49 and Kretz et al. Res. 17:5409.

The E. coli strain C600Hfl (PROMEGA, Madison, WI) was infected with library phage, plated on agar plates, and incubated at 37°C. The phage DNA was transferred to HYBOND-N nylon membranes (AMERSHAM, Arlington Heights, IL) according to the manufacturer's specifications.

Synthetic probes were constructed from overlapping oligonucleotides (A1 probe: 62+63, A2 probe: 52+53; see Table I for their sequences) based on the published dog A1 (RDC7) and

A2a(RDC8) sequences (F Libert, et al,(1989) Science 244:569-572). The oligonucleotides were annealed and filled-in with $a^{32}P$-dCTP (NEN, Wilmington, DE) and Klenow enzyme. The filters were hybridized with the appropriate probe in 5XSSC, 30% formamide, 5XDenhardt's solution, 0.1% SDS, and 0.1mg/ml sonicated salmon sperm DNA at 42°C, overnight. Following hybridization the filters were washed to a final stringency of 6XSSC at 50°C and exposed to X-OMAT AR film (KODAK, Rochester, NY) at -70°C. The resulting positives were plaque purified by two additional rounds of plating and hybridization. Insert DNA was excised with NotI and ligated into NotI digested pBLUESCRIPT II KS+ (STRATAGENE, La Jolla, CA). (Genebank # 52327) DNA sequences were determined by the SEQUENASE protocol (USBC, Cleveland, OH). See Tabor and Richardsaon, J. Biol. Chem. 264 pp 6447-6458. Two clones were isolated in these screens. The human ventricle A1 cDNA (hva1-3a) and human ventricle A2a cDNA (hva2-13) contain portions of coding sequences for proteins homologous to the reported dog A1 and A2a cDNAs, respectively. The coding region of the human A1 clone corresponds to nucleotides 482 through 981 (Figure 2) and is 92% identical to the dog A1 sequence at the nucleotide level. The coding region of the human A2a clone corresponds to nucleotides 497 through 1239 (Figure 4), and is 90% identical to the dog A2a sequence at the nucleotide level.

STEP B:

The human ventricle A1 adenosine receptor partial cDNA (hvA1-3a) is a 543 bp NotI fragment containing 23 bp 3' untranslated sequence and is 460 bp short of the initiation methionine based on sequence homology to the dog A1 cDNA. A modification of the 5' RACE (rapid amplification of cDNA ends) method (MA Frohman et al,(1988), Proc. Natl. Acad. Sci. USA, 85:8998-9002) was used to generate the 5' coding region of the cDNA. First strand cDNA was synthesized from 1μg of the human ventricle poly(A)$^+$ RNA in a total volume of 40ml containing 50mM Tris, pH 8.0, 140mM KCl, 10mM $MgCl_2$, 10mM DTT, 15mM each dNTP, 20 units RNasin (PROMEGA, Madison, WI), 20pmol human primer 79 (see Table I), and 9.2 units AMV reverse transcriptase at 37°C for 2 hrs. The reaction was then diluted to 120 μl with 0.5 mM Tris, pH 7.6/0.05 mM EDTA and passed through a SEPHACRYL S-300 SPUN COLUMN (PHARMACIA, Piscataway, NJ). The product in the column effluent was polyadenylated in 100mM potassium cacodylate, pH 7.2, 2mM $CoCl_2$, 0.2mM DTT, 0.15mM dATP, and 14 units terminal deoxynucleotidyl transferase in a total volume of 31μl for 10 min. at 37°C. The reaction was terminated by heating at 65°C for 15 min. and then diluted to 500 ml with 10 mM Tris, pH 8.0/1 mM EDTA (TE).

Ten μl of the poly(A)-tailed first strand cDNA was used as template in a primary PCR amplification reaction according to the GENEAMP protocol (PERKIN ELMER CETUS, Norwalk, CT; see Saiki et al. (1988) Science 239:487-491) containing 10pmol primer 70, 25pmol primer 71, and 25pmol human primer 80 (see table I) in a total volume of 50 ml. Primer 70 is 5'-gactcgagtcgacatcga(t)$_{17}$, primer 71 is 5'-gactcgagtcgacatcga, and both are based on MA Frohman, et al (1988), Proc. Natl. Acad. Sci. USA, 85:8998-9002. One cycle of PCR was performed of 1 min at 95°C, 2 min at 50°C, 40 min at 72°C, followed by 40 cycles of 40 sec at 94°C, 2 min at 56°C, 3 min at 72°C. The primary PCR amplification reaction product was electrophoresed through a 1.4% agarose gel and an area corresponding to approximately 600 bp was excised. The gel slice was melted and 1 μl was used as

template in a secondary PCR amplification reaction containing 100pmol primer 71 and human primer 81 (see Table I) for 30 cycles of 1 min at 94°C, 2 min at 56°C, 3 min at 72°C. The secondary PCR amplification product was digested with EcoRI and SalI and electrophoresed on a 1.4% agarose gel. An area corresponding to 500-600bp was excised and ligated into EcoRI/SalI digested pBLUESCRIPT II KS+ (STRATAGENE, La Jolla, CA). The sequence of the 515 bp PCR product (5'HVA1-9) was determined by the SEQUENASE protocol (USBC, Cleveland, OH). The partial human ventricle A1 cDNA and the PCR product contain overlapping sequence and represent the complete coding region for the human A1 receptor, including 14 and 23 bp of 5' and 3' untranslated sequences, respectively. The sequence of the human A1 adenosine receptor cDNA so identified, is shown in Figure 2.

STEP C:

A probe was generated by Klenow enzyme extension, including a^{32}P-dCTP, of annealed oligonucleotides 62 and 63, and used to screen a human kidney cDNA library (CLONTECH, Palo Alto, CA). E. coli strain C600hfl (PROMEGA, Madison, WI) was infected with library phage and grown overnight on agar plates at 37°C. Phage DNA was transferred to HYBOND-N nylon filters according to the manufacturer's protocol (AMERSHAM, Arlington Heights, IL). The probe was incubated with the filters in 750mM NaCl, 75mM sodium citrate, 30% formamide, 0.1% sodium dodecyl sulfate, 0.5mg/mL polyvinylpyrrolidone, 0.5mg/mL bovine serum albumin, 0.5mg/mL Ficoll 400, and 0.1mg/mL salmon sperm DNA, at 42°C overnight. The filters were washed in 0.9M NaCl and 90mM sodium citrate at 50°C. A positively hybridizing phage (hkA1-14), was identified and purified by replating and screening with the probe twice more. The final phage plaque was transferred to 0.5 mL 50mM Tris, pH 7.5, 8mM $MgSO_4$, 85 mM NaCl, 1mg/mL gelatin, and 1 µL of a 1:50 dilution in water of the phage stock was used as template for PCR amplification. 50 pmol each of 1amL and 1amR (Table I) oligonucleotide primers were included, and subjected to 30 cycles of 40 sec at 94°C, 1 min at 55°, 3 min at 72°,

then a final 15 min at 72°, according to the GENEAMP protocol (PERKIN ELMER CETUS, Norwalk, CT). A 2.0 kb product was identified by agarose gel electrophoresis, and this was subcloned into the EcoRI site of pBLUESCRIPT II KS+ (STRATAGENE, La Jolla, CA). Sequence analysis by the SEQUENASE protocol (USBC, Cleveland, OH) demonstrated that this cDNA was homologous to the reported dog A1 clone. SmaI and EcoRI digestion released a DNA fragment containing coding sequence from base pair 76 through the translation STOP codon (Figure 2) that is identical to the human ventricle A1 cDNA sequence (clones hva1-3a and 5'hva1-9). This fragment was used in construction of the full length coding sequence (see below). The human kidney cDNA also includes about 900 bp of 3' untranslated sequence.

<u>STEP D:</u>

The human ventricle A2a adenosine receptor partial cDNA (hvA2-13) is a 1.6 kb NotI fragment containing approximately 900 bp 3' untranslated sequence and is 496 bp short of the initiation methionine based on sequence homology to the dog A2a cDNA clone. Two consecutive rounds of 5' RACE were utilized to generate the 5' coding region of the cDNA. First strand cDNA was synthesized from 1 µg of the human ventricle poly(A)$^+$ RNA in a total volume of 40 ml containing 50mM Tris, pH 8.0, 140mM KCl, 10mM $MgCl_2$, 10mM DTT, 15mM each dNTP, 20 units RNasin, 20pmol human primer 68 or 74 (for 1st or 2nd round RACE respectively), and 9.2 units AMV reverse transcriptase at 37°C for 2 hrs. The reaction was then diluted to 120ml with 0.5 mM Tris, pH 7.6/0.05 mM EDTA and passed through a SEPHACRYL S-300 SPUN COLUMN. The products in the column effluents were polyadenylated in 100mM potassium cacodylate, pH 7.2, 2 mM $CoCl_2$, 0.2 mM DTT, 0.15 mM dATP, and 14 units terminal deoxynucleotidyl transferase in a total volume of 31 µl for 10 min. at 37°C. The poly(A) tailing reaction was terminated by heating at 65°C for 15 min. and then diluted to 500 µl with TE.

Five or 10 µl (for 1st or 2nd round RACE respectively) of the poly(A) tailed first strand cDNA was used as template in the PCR amplification reaction according to the GENEAMP protocol containing 10pmol primer 70, 25 pmol primer 71 (primer 70 and 71 sequences are given above), and 25 pmol human primer 69 or 75 (1st or 2nd round RACE respectively; see Table I) in a total volume of 50 µl. One cycle of PCR was performed of 1 min at 95°C, 2 min at 50°C, 40 min at 72°C, followed by 40 cycles of 40 sec at 94°C, 2 min at 56°C, 3 min at 72°C. The PCR amplification products were digested with EcoRI and SalI and electrophoresed on a 1.4% agarose gel. Areas corresponding to 200-400 bp were excised and ligated into EcoRI/SalI digested pBLUESCRIPT II KS+ (STRATAGENE, La Jolla, CA). The sequences of the two A2a PCR products, the 332 bp 1st round RACE product (5'hvA2-14) and the 275 bp 2nd round RACE product (5'hvA2-29) were determined by the SEQUENASE (USBC, Cleveland, OH) protocol. By sequence homology comparisons with the dog A2a adenosine receptor cDNA sequence, the 1st round RACE product (5'hvA2-14) was 258 bp short of the initiation methionine and the second round RACE product (5'HVA2-29) was determined to extend 1bp upstream of the initiation methionine. The human ventricle A2a partial cDNA clone (hvA2-13) and the human A2a PCR products (5'hvA2-14 and 5'hva2-29) contain overlapping sequence and together represent the complete coding sequence for the human adenosine A2a receptor, and include 1 bp and 0.8 kb of 5' and 3' untranslated sequence, respectively. The sequence of the human A2a adenosine receptor is shown in Figure 4.

STEP E:

A double-stranded DNA probe was generated by Klenow enzyme extension, including a^{32}P-dCTP, of annealed oligonucleotides 66 and 67, and used to screen a human striata cDNA library (STRATAGENE, La Jolla, CA). The oligonucleotide sequence was based on a region of the human ventricle A2a cDNA sequence. E. coli strain XL1-blue (STRATAGENE, La Jolla, CA) cells were infected with library phage and grown overnight on agar plates at 37°C. Phage DNA was transferred to HYBOND-N nylon filters according to the manufacturer's protocol (AMERSHAM, Arlington Heights, IL). The probe was incubated with the filters in 750 mM NaCl, 75 mM sodium citrate, 10% formamide, 0.5% sodium dodecyl sulfate, 0.5 mg/mL polyvinylpyrrolidone, 0.5 mg/mL bovine serum albumin, 0.5 mg/mL Ficoll 400, and 0.02 mg/mL salmon sperm DNA, at 42°C overnight. The filters were washed in 0.9 M NaCl and 90 mM sodium citrate at 50°C. A positively hybridizing phage (hbA2-22A) was identified and purified by replating and screening with the probe twice more, and subcloned into the plasmid pBLUESCRIPT SK- by the manufacturer's protocol (STRATAGENE, La Jolla, CA). See Short et al. (1988) Nucl. Acids Res. 16:7583-7600; Sorge (1988) Stratagies 1:3-7. The human brain A2a adenosine receptor cDNA (hbA2-22A) spans bp 43 of the A2 coding sequence (Figure 4) through the translation STOP codon, and includes about 900 bp of 3' untranslated sequence. The sequence of this human brain A2a cDNA is identical to the human ventricle A2a adenosine receptor cDNA (hvA2-13, 5'hvA2-14 and 5'hvA2-29).

STEP F:

A double-stranded DNA probe was generated by Klenow enzyme extension of annealed oligonucleotides 129 and 130, including a^{32}P-dCTP, and used to screen a human frontal cortex cDNA library (STRATAGENE, La Jolla, CA). The oligonucleotide sequence was based on a region of the human A2a and A1 cDNA sequence. E. coli strain XL-1 blue (STRATAGENE, La Jolla, CA) cells were infected with library phage and grown overnight at 37°C. Phage DNA was

transferred to HYBOND-N nylon filters according to the manufacturer's protocol (AMERSHAM, Arlington Heights, IL). The probe was incubated with the filters in 750 mM NaCl, 75 mM sodium citrate, 10% formamide, 0.5% sodium dodecyl sulfate, 0.5 mg/mL polyvinyl-pyrrolidone, 0.5 mg/mL bovine serum albumin, 0.5 mg/mL Ficoll 400, and 0.02 mg/mL salmon sperm DNA, at 42°C overnight. The filters were washed in 0.9 M NaCl and 90 mM sodium citrate at 50°C. A positively hybridizing phage (hb-32c), was identified and purified by replating and screening with the probe twice more. The insert was subcloned to the plasmid pBLUESCRIPT SK- according to the manufacturer's protocol (STRATAGENE, La Jolla, CA). Sequence analysis by the SEQUENASE protocol (USBC, Cleveland, OH) demonstrated a complete open reading frame coding for amino acid sequence homologous to both of the previously isolated human A1 and A2a clones. This homologous adenosine receptor subtype cDNA is the A2b subtype having the sequences in Figures 5 and 6. A 1.3 kb SmaI-XmnI fragment was ligated into the SmaI site of pSVL (PHARMACIA, Piscataway, NJ), giving the full length coding sequence of the A2b adenosine receptor in a plasmid suitable for its expression in COS and CHO cells. See Sprague et al. (1983) J. Virology 45:773; Templeton and Eckhart (1984) Mol. Cell Biol. 4:817.

Table I:

Sequences and directions of the primers used in the isolation of cDNA's and construction of expression plasmids, along with the positions in the clones upon which the sequences are based. Dog A1 and A2a cDNA clones are from F. Libert, et al, (1989) Science 244:569-572. Primers LamL and LamR are based on the sequence of λgt10 (T.V. Hyunh, et al. (1985) DNA Cloning: A Practical Approach, Vol 1, D. Glover, ed, IRL Press, Oxford). The A2b adenosine receptor subtype encoded by the clone hb32C was determined to be the A2b adenosine receptor subtype on the basis of the binding profile of the adenosine receptor agonist NECA and affinities for adenosine receptor

antagonists measured on membranes prepared from pSVLhb32C transfected COS7, CHO or HEK 293 cells.

name	sequence	position	clone	direction
52	ATTCGCAGCCACGTCCTGA-GGCGGCGGGAGCCCTTCAA-AGCAGGTGGCACCAGTGCC-CGC (SEQ ID NO. 1)	1201-1260	dog A2a	sense
53	GCGGAGGCTGATCTGCT-CTCCATCACTGCCATGAG-CTGCCAAGGCGCGGGCAC-TGGTGCC (SEQ. ID NO. 2)	1305-1246	dog A2a	antisense
62	TCCAGAAGTTCCGGGTCA-CCTTCCTTAAGATCTGGAA-TGACCACTTCCGCTGCCAGC-CCA (SEQ. ID NO. 3)	958-1017	dog A1	sense
63	AGTCGTGGGGCGCCTCCT-CTGGGGGGTCCTCGTCGAC-GGGGGGCGTGGGCTGGCAG-CGGA (SEQ ID NO. 4)	1062-1003	dog A1	antisense
66	GCCTCTTTGAGGATGTGG-TCCCCATGAACTACATGGT-GTACTTCA (SEQ ID NO. 5)	500-542	5'hvA2-14	sense
67	GCAGGGGCACCAGCACACA-GGCAAAGAAGTTGAAGTAC-ACCATGT (SEQ ID NO. 6)	572-528	5'hva2-14	antisense

name	sequence	position	clone	direction
68	TCGCGCCGCCAGGAAGAT (SEQ ID NO 7)	616-599	hva2-13	antisense
69	TATATTGAATTCTAGACAC-CCAGCATGAGC (SEQ ID NO.8)	591-574	hva2-13	antisense
74	TCAATGGCGATGGCCAGG (SEQ ID NO. 9)	303-286	5'hva2-14	antisense
75	TATATTGAATTCATGGA-GCTCTGCGTGAGG-(SEQ ID NO. 10)	276-259	5'hva2-14	antisense
79	GTAGACCATGTACTCCAT (SEQ ID NO. 11)	560-543	hva1-3a	antisense
80	TATATTGAATTCTGACCT-TCTCGAACTCGC-(SEQ ID NO. 12)	537-521	hva1-3a	antisense
81	ATTGAATTCGATCACGGG-CTCCCCCATGC-(SEQ ID NO. 13)	515-496	hva1-3a	antisense
129	ATGGAGTACATGGTCTAC-TTCAACTTCTTTGTGTGGG-TGCTGCCCCCGCT-(SEQ ID NO. 14)	---	---	sense

name	sequence	position	clone	direction
130	GAAGATCCGCAAATAGACA-CCCAGCATGAGCAGAAGCG-GGGGCAGCACCC (SEQ ID NO. 15)	---	---	antisense
131	CCCTCTAGAGCCCAGCCTGT-GCCCGCCATGCCCATCATGG-GCTCC (SEQ ID NO. 16)	2-19 1-14	5'hva2-29 5'hva1-9	sense
lamL	CCCACCTTTTGAGCAAGTTC (SEQ ID NO. 17)	---	λt10	---
lamR	GGCTTATGAGTATTTCTTCC (SEQ ID NO. 18)	---	λt10	---
207	CCCAAGCTTATGAAAGCCAA CAATACC (SEQ ID NO. 27)			
208	TGCTCTAGACTCTGGTATCT TCACATT (SEQ ID NO. 28)			

EXAMPLE 2

Human A1 adenosine receptor expression construct:

To express the human adenosine receptor cDNA in COS, CHO and HEK 293 cells, the 118bp SalI-SmaI fragment of the human ventricle A1 PCR product (5'HVA1-9) was ligated together with the 1.8 SmaI-EcoRI fragment of the human kidney A1 adenosine receptor cDNA (hkA1-14) and the 3.0 kb SalI-EcoRI fragment of

pBLUESCRIPT II KS+, resulting in a plasmid containing the contiguous full length coding sequence for the human A1 adenosine receptor cDNA and some 5' and 3' untranslated sequence. This plasmid was digested first with EcoRI, the resulting ends were filled in by Klenow enzyme extension and then the plasmid was digested with XhoI to release a fragment of 1.9 kb containing the full length human A1 adenosine receptor cDNA. The fragment was subcloned into the expression vector pSVL (PHARMACIA) which had been digested with XhoI-SmaI.

Human A2a adenosine receptor expression construct:

To express the human A2a adenosine receptor cDNA in COS, CHO or HEK 293 cells, a contiguous A2a cDNA sequence was constructed before subcloning into the expression vector, pSVL. Primer 131, containing an XbaI recognition site, 14 bp of 5' untranslated sequence of human A1 adenosine receptor cDNA, and the first 18 bp of human A2a adenosine receptor cDNA coding sequence was used with primer 75 in PCR with 1 ng of the plasmid containing the human ventricle A2a 2nd round RACE product (5'hvA2-29) as template. Twenty-five cycles of 40 sec at 94°C, 1 min at 55°C, and 3 min at 72°C, then a final incubation of 15 min at 72°C, with 1 ng of plasmid template and 50 pmol of each primer in a volume of 50 μL according to the GENEAMP protocol (PERKIN ELMER CETUS, Norwalk, CT), resulted in the expected 302 bp product determined by agarose gel electrophoresis. The 172 bp XbaI-EagI digestion product of this DNA fragment was ligated together with 1125 bp EagI-BglII digestion product of the human striata A2a adenosine receptor cDNA (hbA2-22A) and XbaI-SmaI digested pSVL (PHARMACIA), generating the full length human A2a adenosine receptor cDNA coding sequence in a plasmid suitable for its expression in COS, CHO or HEK 293 cells.

Mammalian cell expression:

COS7 cells (ATCC #1651-CRL) were grown in complete medium, Dulbecco's modified Eagles's medium, DMEM (GIBCO, Grand Island, NY) containing 10% fetal bovine serum, 100U/mL

penicillin-streptomycin and 2 mM glutamine, in 5% CO_2 at 37°C. Transient transfection of COS7 cells was performed by the $CaPO_4$ method (Graham,F.L. and Van Der Erb, A.J. (1973) Virology 52:456-567) using the Mammalian Transfection Kit (STRATAGENE). See Chen and Okayama Mol. Cell Biol. 7:2745-2752. Plasmid DNA (15 mg) was precipitated with 125 mM $CaCl_2$ in BBS (N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid buffered saline) at room temperature for 30 minutes. The DNA precipitate was added to the COS7 cells and incubated for 18h in 5% CO_2 at 37°C. The precipitate was removed and the cells were washed twice with serum free DMEM. Cells were incubated in complete medium in 5% CO_2 at 37°C for 48 h prior to the binding assay.

Stable expression in CHO or HEK 293 cells:

To establish stable cell lines, CHO or HEK 293 cells were co-transfected with 20 μg of pSVL containing the adenosine receptor cDNA and 1mg of pWLneo (STRATAGENE) containing the neomycin gene. See Southern and Berg (1982) J. Mol. App. Gen. 1:327-341. Transfection was performed by the $CaPO_4$ method. DNA was precipitated at room temperature for 30 minutes, added to the CHO cells and incubated 18h in 5% CO_2 at 37°C. The precipitate was removed and the cells were washed twice with serum free DMEM. Cells were incubated for 24h in 5% CO_2 at 37°C, replated in 24-well dishes at a dilution of 1:10, and incubated an additional 24h before adding selection medium, DMEM containing 10% fetal bovine serum, 100U/mL penicllin-streptomycin, 2 mM glutamine and 0.5 mg/mL G418 (GIBCO). Transfected cells were incubated at 5% CO_2, 37°C until viable colonies were visible, approximately 14-21 days. Colonies were selected and propagated. The cell clone with the highest number of human adenosine receptors was selected for subsequent application in the binding assay.

EXAMPLE 3

Binding studies:

Membranes were prepared from transiently transfected COS7 cells 48 h after transfection or from G418-selected stably transfected CHO or HEK 293 cells. Cells were harvested in 1 mM EDTA in phosphate buffered saline and centrifuged at 2000 x g for 10 minutes. The cell pellet was washed once with phosphate buffered saline. The cell pellet was resuspended in 2 mL of 5 mM Tris, pH 7.6/ 5mM $MgCl_2$. Membranes were prepared from the cells by freeze-thaw lysis in which the suspension was frozen in a dry ice/ethanol bath and thawed at 25°C twice. The suspension was homogenized after adding an additional 2 mL of 5 mM Tris, pH 7.6/5 mM $MgCl_2$, in a glass dounce homogenizer with 20 strokes. The membranes were pelleted at 40,000 x g at 4°C for 20 minutes. The membrane pellet was resuspended at a protein concentration of 1-2 mg/mL in binding assay buffer, 50 mM Tris, pH 7.6/10 mM $MgCl_2$. Protein concentration was determined by the method of Bradford ((1976) Anal. Biochem. 72: 248-250). Before the binding assay was performed, the membranes were incubated with adenosine deaminase (BOEHRINGER MANNHEIM), 2 U/mL for 30 minutes at 37°C. Saturation binding of [^{3}H]-cyclohexyladenosine (CHA) was performed on membranes prepared from pSVLA1 transfected COS7 or CHO cells.

Membranes (100μg) were incubated in the presence of 0.2 U/mL adenosine deaminase with increasing concentrations of CHA (NEN, 32 Ci/mmol) in the range of 0.62 - 30 nM for 120 minutes at 25°C in a total volume of 500 μL. The binding assay was terminated by rapid filtration and three washes with ice-cold 50 mM Tris,pH 7.6/10 mM $MgCl_2$ on a SKATRON CELL HARVESTER equipped with a receptor binding filtermat (SKATRON INSTRUMENTS, INC). Non-specific binding was determined in the presence of 100 μM N^6-cyclopentyladenosine (CPA). Bound radioactivity was measured by scintillation counting in READY SAFE SCINTILLATION COCKTAIL (BECKMAN). For competition binding experiments, membranes were

incubated with 5 nM [^{3}H]-CHA and various concentrations of A1 adenosine receptor agonists. Saturation binding of [^{3}H] CGS-21680 was performed on membranes prepared from pSVLA2a transfected COS7 cells. Membranes (100μg) were incubated in the presence of 0.2 U/mL adenosine deaminase with increasing concentrations of CGS21680 (NEN, 48.6 Ci/mmol) in the range of 0.62 -80 nM for 90 minutes at 25°C in a total volume of 500 μL. The binding assay was terminated by rapid filtration with three washes with ice-cold 50 mM Tris, pH 7.6/10 mM $MgCl_2$ on a Skatron cell harvester equipped with a receptor binding filtermat (SKATRON INSTRUMENTS, INC). Non-specific binding was determined in the presence of 100μM CPA. Bound radioactivity was measured by scintillation counting in READY SAFE LIQUID SCINTILLATION COCKTAIL (BECKMAN). For competition binding experiments, membranes were incubated with 5nM [^{3}H]-CGS21680 and various concentrations of A2 adenosine receptor agonists.

Saturation binding of [^{3}H]5'-N-ethylcarboxamidoadenosine (NECA) was performed on membranes (100 μg) prepared from pSVLhb32C (A2b) transfected COS7 cells in the presence of adenosine deaminase with increasing concentrations of NECA (NEN, 15.1Ci/mmol) in the range of 1.3-106 nM for 90 minutes at 25°C in a total volume of 500 μL. The assay was terminated by rapid filtration and three washes with ice-cold binding buffer on a cell harvester equipped with a receptor binding filtermat (SKATRON INSTRUMENTS, INC). Bound radioactivity was measured by scintillation counting. Non-specific binding was measured on membranes prepared from non-transfected COS7 cells. For competition binding experiments, membranes from transfected cells were incubated with 10 nM [^{3}H]NECA and varying concentrations of adenosine receptor antagonists.

EXAMPLE 4

The human A3 adenosine receptor was cloned from a human striata cDNA library. Oligonucleotide probes were designed based on the rat A3 sequence of Zhou et al., Proc. Natl. Acad. Sci. 89, 7432 (1992). The complete sequence of the human A3 adenosine receptor was determined and the protein sequence deduced. The cloned human A3 adenosine receptor is expressed in a heterologous expression system in COS, CHO and HEK 293 cells. Radiolabeled adenosine receptor agonists and antagonists are used to measure the binding properties of the expressed receptor. Stable cell lines can be used to evaluate and identify adenosine receptor agonists, antagonists and enhancers.

STEP A:

A synthetic probe homologous to the rat A3 adenosine receptor was generated using the polymerase chain reaction (PCR). Three µl of rat brain cDNA was used as template in a PCR amplification reaction according to the GENEAMP protocol (PERKIN ELMER CETUS, Norwalk, CT) containing 50 pmol of primers 207 (5'-cccaagcttatgaaagccaacaatacc) (SEQ. ID NO: 27) and 208 (5'-tgctctagactctggtatcttcacatt) (SEQ. ID NO: 28) in a total volume of 50 ml. Primers 207 and 208 are based on the published rat A3 adenosine receptor sequence (Zhou, et al, (1992), Proc. Natl. Acad. Sci. USA, 89:7432-7406). Forty cycles of 40 sec at 94°C, 1 min at 55°C, 3 min at 72°C were performed and the resulting 788 bp fragment was subcloned into HindIII-XbaI digested pBLUESCRIPT II KS+ (STRATAGENE, La Jolla, CA). The sequence was verified by the SEQUENASE protocol (USBC, Cleveland, OH).

STEP B:

The 788 bp PCR fragment was labeled with a^{32}P-dCTP using the MULTIPRIME DNA LABELLING SYSTEM (AMERSHAM, Arlington Heights, IL) and used to screen a human striata cDNA library

(STRATAGENE, La Jolla, CA). E. coli strain XL-1 Blue (STRATAGENE, La Jolla, CA) cells were infected with library phage and grown overnight at 37°C. Phage DNA was transferred to HYBOND-N nylon filters according to the manufacturer's protocol (AMERSHAM, Arlington Heights, IL). The probe was incubated with the filters in 5 X SSC, 30% formamide, 5 X Denhardt's solution, 0.5% sodium dodecyl sulfate, and 50 mg/ml sonicated salmon testis DNA. The filters were washed in 2 X SSC at 55°C. A positively hybridizing phage (HS-21a) was identified and plaque purified by two additional rounds of plating and hybridization. The insert was subcloned to the plasmid pBLUESCRIPT II SK- according to the manufacturer's protocol (STRATAGENE, La Jolla, CA). Upon sequence analysis using the SEQUENASE protocol (USBC, Cleveland, OH) it was determined that clone HS-21a contained the complete open reading frame corresponding to the human homolog of the rat A3 adenosine receptor. The coding region of the human A3 adenosine receptor cDNA is 78% identical to the rat sequence at the nucleotide level and contains 265 bp and 517 bp of 5' and 3' untranslated sequence, respectively. The 1.7 kb fragment was excised using sites present in the multiple cloning site of pBLUESCRIPT II SK- (STRATAGENE, La Jolla, CA) and subcloned into XhoI/SacI digested pSVL (PHARMACIA, Piscataway, NJ) for its expression in COS and CHO cells.

EXAMPLE 5

Mammalian cell expression:

COS7 cells (ATCC #1651-CRL) were grown in complete medium, Dulbecco's modified Eagles's medium, DMEM (GIBCO, Grand Island, NY) containing 10% fetal bovine serum, 100U/mL penicillin-streptomycin and 2mM glutamine, in 5% CO_2 at 37°C. Transient transfection of COS7 cells was performed by the $CaPO_4$ method (Graham, F.L. and Van Der Erb, A.J. (1973) Virology 52:456-567) using the Mammalian Transfection Kit (STRATAGENE). Plasmid DNA (15 mg) was precipitated with 125 mM $CaCl_2$ in BBS (N,N-bis(2-

hydroxyethyl)-2-aminoethanesulfonic acid buffered saline) at room temperature for 30 minutes. The DNA precipitate was added to the COS7 cells and incubated for 18 h in 5% CO_2 at 37°C. The precipitate was removed and the cells were washed twice with serum free DMEM. Cells were incubated in complete medium in 5% CO_2 at 37°C for 48 h prior to the binding assay.

Stable expression in CHO cells:

To establish stable cell lines, CHO cells were cotransfected with 20 µg of pSVL containing the adenosine receptor cDNA and 1 µg of pWLneo (STRATAGENE) containing the neomycin gene. Transfection was performed by the $CaPO_4$ method. DNA was precipitated at room temperature for 30 minutes, added to the COS7 cells and incubated 18 h in 5% CO_2 at 37°C. The precipitate was removed and the cells were washed twice with serum free DMEM. Cells were incubated for 24 h in 5% CO_2 at 37°C, replated in 24-well dishes at a dilution of 1:10, and incubated an additional 24 h before adding selection medium, DMEM containing 10% fetal bovine serum, 100U/mL penicillin-streptomycin, 2 mM glutamine and 1.0 mg/mL G418 (GIBCO). Transfected cells were incubated at 5% CO_2, 37°C until viable colonies were visible, approximately 14-21 days. Colonies were selected and propagated. The cell clone with the highest number of human adenosine receptors was selected for subsequent application in the binding assay.

EXAMPLE 6

Binding assay:

Membranes were prepared from transiently transfected COS7 cells 48 h after transfection or from G418-selected stably transfected CHO or HEK 293 cells. Cells were harvested in 1 mM EDTA in phosphate buffered saline and centrifuged at 2000 x g for 10 minutes. The cell pellet was washed once with phosphate buffered saline. The cell pellet was resuspended in 2 mL of 5 mM Tris, pH 7.6/

$5mM\ MgCl_2$. Membranes were prepared from the cells by freeze-thaw lysis in which the suspension was frozen in a dry ice/ethanol bath and thawed at 25°C twice. The suspension was homogenized after adding an additional 2 mL of 5 mM Tris, pH 7.6/ 5mM $MgCl_2$, in a glass dounce homogenizer with 20 strokes. The membranes were pelleted at 40,000 x g at 4°C for 20 minutes. The membrane pellet was resuspended at a protein concentration of 1-2 mg/mL in binding assay buffer, 50 mM Tris, pH 7.6/10 mM $MgCl_2$. Protein concentration was determined by the method of Bradford ((1976) Anal. Biochem. 72: 248-250). Before the binding assay was performed, the membranes were incubated with adenosine deaminase (BOEHRINGER MANNHEIM), 2U/mL for 30 minutes at 37°C. Saturation binding of $[^{125}I]$-N^6-aminobenzyl-adenosine (^{125}I-ABA) or $[^{125}I]$-N^6-2-(4-amino-3-iodophenyl)ethyl-adenosine (APNEA) was performed on membranes prepared from pSVLA3 transfected COS7 cells. Membranes (100 μg) were incubated in the presence of 0.2U/mL adenosine deaminase with increasing concentrations of ^{125}I-ABA in the range of 0.1-30 nM for 120 minutes at 25°C in a total volume of 500 μL. The binding assay was terminated by rapid filtration and three washes with ice-cold 50 mM Tris, pH 7.6/10 mM $MgCl_2$ on a Skatron cell harvester equipped with a receptor binding filtermat (SKATRON INSTRUMENTS, INC). Non-specific binding was determined on non-transfected cells. Bound radioactivity was measured by scintillation counting in Ready Safe Scintillation Cocktail (BECKMAN).

EXAMPLE 7

In vitro transcription and oocyte expression:

The 1.3 kb XhoI-BamHI fragment of the pSVL expression construct (described in Example 2) containing the full length human A2a adenosine receptor coding sequence was ligated into SalI-SpeI digested pGEMA (Swanson, et al, (1990) Neuron 4:929-939). The resulting plasmid, pGEMA2, was linearized with NotI, forming a template for in vitro transcription with T7 RNA polymerase. The

homologous adenosine receptor subtype cDNA in pBluescript SK- was used as a template for in vitro transcription by T3 polymerase after removal of most of the 5' untranslated region, with the exception of 20 bp, as a 0.3 kb SmaI fragment. The K^+ channel cDNA, Kv3.2b was employed as a negative control in the cAMP accumulation assay. The generation of Kv3.2b RNA was described by Luneau, et al, ((1991) FEBS Letters 1:163-167). Linearized plasmid templates were used with the STRATAGENE mCAP kit according to the manufacturer's protocol, except that the SP6 RNA polymerase reaction was performed at 40°C. Oocytes were harvested from mature female *Xenopus laevis*, treated with collagenase, and maintained at 18°C in ND96 medium (GIBCO) supplemented with 1 mM sodium pyruvate and 100 mg/mL gentamycin. Fifty nanoliters (10 ng) of RNA diluted in H_2O was injected and oocytes were incubated at 18°C for 48 hours.

EXAMPLE 8

cAMP accumulation assay in oocytes:

Oocytes injected with either human adenosine receptor transcript or the Kv3.2b transcript were transferred to fresh medium supplemented with 1 mM of the phosphodiesterase inhibitor, Ro 20-1724 (RBI, Natick, MA) and 1 mg/mL bovine serum albumin incubated for 30 minutes and transferred to an identical medium with or without the agonist adenosine (10 mM) for an additional 30 minutes at room temperature. Groups of 5-10 oocytes were lysed by transfer to ND96/100 mM HCl/1 mM Ro 20-1724 in microfuge tubes, shaken, incubated at 95°C for 3 min, and centrifuged at 12000 g for 5 min. Supernatants were stored at -70°C before cAMP measurements. Cyclic AMP levels were determined by radioimmunoassay (RIANEN kit, DuPont/NEN) using the acetylation protocol. The adenosine receptor antagonist, 8-(p-sulfophenyl)theophylline (100 µM) was utilized to inhibit the cAMP response induced by adenosine in oocytes expressing the adenosine receptors.

EXAMPLE 9

cAMP accumulation in stable CHO cell lines:

The changes in cAMP accumulation can alternatively be measured in stably transfected CHO cells expressing the human adenosine receptor subtypes. CHO cells are washed twice in phosphate buffered saline (PBS) and detached in 0.2% EDTA in PBS. The cells are pelleted at 800 rpm for 10 min and resuspended in KRH buffer (140 mM NaCl/5 mM KCl/2 mM $CaCl_2$/1.2 mM $MgSO_4$/1.2 mM KH_2PO_4/6 mM glucose/25 mM Hepes buffer, pH 7.4). The cells are washed once in KRH buffer and resuspended at 10^7 cells/mL. The cell suspension (100 µL) is mixed with 100 µL of KRH buffer containing 200 mM Ro 20-1724 and incubated at 37°C for 10 minutes. Adenosine (10 mM), NECA or CPCA was added in 200 µL KRH buffer containing 200 µM Ro 20-1724 and incubated at 37°C for 20 minutes. After the incubation, 400 mL of 0.5 mM NaOAc (pH 6.2) was added and the sample was boiled for 20 minutes. The supernatant was recovered by centrifugation for 15 minutes and stored at -70°C. cAMP levels were determined by radioimmunoassay (RIANEN kit, DuPont/NEN) using the acetylation protocol. The effect of antagonists on cAMP accumulation are measured by preincubation for 20 minutes before adding adenosine.

EXAMPLE 10

Expression Construct and Transfection

The 1.7 kb HS-21a cDNA (A3) was subcloned as a SalI-BamHI fragment into the expression vector pCMV5 (Mumby, S.M., Heukeroth, R.O., Gordon, J.I.and Gilman, A.G. (1990) Proc. Natl. Acad. Sci. USA **87**, 728-732.) creating the vector pCMV5-A3. CHO or HEK 293 cells stably expressing the human HS-21a cDNA were prepared by co-transfection of 15 µg pCMV5-A3 and 1 µg pWLneo (Stratagene) using the calcium phosphate method. Stable cell lines were also generated using EBV based mammalian expression vectors, pREP (INVITROGEN). Neomycin resistant colonies were selected in 1

mg/mL G418 (GIBCO). Stable colonies were screened for expression of HS-21a by ^{125}I-ABA binding.

EXAMPLE 11

Binding Studies

Membranes were prepared from stable CHO cell lines in 10 mM Hepes, pH 7.4 containing 0.1 mM benzamidine and 0.1 mM PMSF as described (Mahan, L.C., et al., (1991) Mol. Pharmacol. **40**, 1-7). Pellets were resuspended in 5 mM Hepes, pH 7.4/5 mM $MgCl_2$/0.1 mM benzamidine/0.1 mM PMSF at a protein concentration of 1-2 mg/mL and were incubated with adenosine deaminase (Boehringer Mannheim), 2U/mL at 37 °C for 20 minutes. Saturation binding of ^{125}I-ABA was carried out on 50 mg of membranes for 120 minutes at 25 °C in a total volume of 100 µL. The assay was terminated by rapid filtration and three washes with ice-cold binding buffer on a Skatron harvester equipped with a receptor binding filtermat (Skatron Instruments, INC). The specific activity of ^{125}I-ABA, initially 2,200 Ci/mmol, was reduced to 100 Ci/mmol with nonradioactive I-ABA for saturation analysis. Nonspecific binding was measured in the presence of 1 mM I-ABA. The K_D and B_{max} values were calculated by the EBDA program (McPherson, G.A. (1983) Computer Programs for Biomedicine **17**, 107-114). Competition binding of agonists and antagonists was determined with ^{125}I-ABA (0.17-2.0 nM, 2000 Ci/mmol). Nonspecific binding was measured in the presence of 400 mM NECA. Binding data were analyzed and competition curves were constructed by use of the nonlinear regression curve fitting program Graph PAD InPlot, Version 3.0 (Graph Pad Software, San Diego). K_i values were calculated using the Cheng-Prusoff derivation (Cheng, Y.C. and Prusoff, H.R. (1973) Biochem. Pharmacol. **22**, 3099-3108.).

The binding properties of the receptor encoded by HS-21a were evaluated on membranes prepared from CHO cells stably expressing the HS-21a cDNA. The radioligand, ^{125}I-APNEA, was previously used to characterize rat A3 adenosine receptors. In preliminary experiments, high non-specific ^{125}I-APNEA binding to

CHO cell membranes was observed which interfered with the measurement of specific binding to expressed receptors. Specific and saturable binding of the adenosine receptor agonist, ^{125}I-ABA was measured on membranes prepared from the stably transfected cells (Figure 11A). The specific binding of ^{125}I-ABA could be prevented by either 1 mM nonradioactive I-ABA or 400 μM NECA. No specific binding of ^{125}I-ABA was measured on membranes prepared from non-transfected CHO cells. The specific binding of ^{125}I-ABA measured in either the presence of 10 μM GTPγS or 100 μM Gpp(NH)p was reduced by 56 and 44% respectively, relative to the specific binding measured in the absence of the uncoupling reagents. These results suggest that ^{125}I-ABA exhibits some agonist activity on the receptor encoded by the HS-21a cDNA expressed in the stable CHO cell line. ^{125}I-ABA binds to membranes prepared from the HS-21a stable CHO cells with a dissociation constant of 10 nM (B_{max}= 258 fmol/mg protein) with a Hill coefficient of 0.99 indicating binding to a single class of high affinity sites (Figure 11B).

The competition of adenosine receptor agonists and antagonists for binding to HS-21a receptors was determined (Figure 12). The K_i values for agonists (top panel) were calculated to be 26 nM for NECA, 34 nM for R-PIA, 89 nM for CPA and 320 nM for S-PIA, resulting in a potency order profile of NECA >R-PIA > CPA > S-PIA. In contrast to the insensitivity of adenosine receptor antagonists reported for the rat A3 adenosine receptor subtype, a number of xanthine antagonists exhibited competition with ^{125}I-ABA for binding to the HS-21a receptor (Figure 12, lower panel). Studies of the sheep A3 adenosine receptor indicated that 8-phenylxanthines substituted in the para-position with acidic substituents are high affinity antagonists. By evaluating additional xanthines in this class I-ABOPX was determined to be the highest affinity antagonist yet reported for A3 adenosine receptors. The K_i values for antagonists were calculated to be 18 nM for I-ABOPX, 55 nM for BW-A1433, 70 nM for XAC and 750 nM for DPCPX, resulting in a potency order profile of I-ABOPX >BW-A1433 > XAC >DPCPX.

EXAMPLE 12

cAMP Studies

Determinations were made on stably transfected CHO cells in suspension as described (Linden et al., (1993) Mol. Pharm. 44:524-532). Supernatants (500 μL) were acetylated and acetylcyclic AMP was measured by automated radioimmunoassay (Hamilton, B.R. and Smith, D.O. (1991) J. Physiol. (Lond.) **432**, 327-341). Antagonist dissociation constants were estimated from pA_2 values as described by Schild (1957) Pharm. Rev. **9**, 242-246).

EXAMPLE 13

Northern Blot Analysis

Human poly(A)+ RNA from different tissue sources (Clontech) is fractionated on a 1% agarose-formaldehyde gel (Sambrook, J., Fritsch, E. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, (Cold Spring Harbor Press, Cold Spring Harbor, NY), transferred to Hybond-N membranes and hybridized in 5XSSPE, 5XDenhardt's, 0.5% SDS, 50 mg/mL sonicated salmon testis DNA, with 30% formamide (for A1, A2a, and A2b) or 50% formamide (for HS-21a) at 42°C. DNA probes corresponding to nucleotides 512-1614, 936-2168, and 321-1540 of accession numbers X68485(A1), X68486(A2a), and X68487(A2b) respectively, and a 1.7 kb SalI-BamHI fragment of HS-21a were labeled with $a^{32}P$-dCTP by the random priming method. Filters were washed under high stringency conditions in 0.1XSSC at 65°C.

EXAMPLE 14

INHIBITION OF TNFα PRODUCTION

STEP A:

Isolation of human peripheral blood mononuclear cells.

Human blood is obtained by venipuncture from healthy donors and collected into tubes containng 20U/mL of heparin sodium salt. The blood is diluted 1:1 with Hanks balanced salts solution containing 20 U/mL Heparin. Peripheral blood mononulear cells (PBMC) are isolated by Ficoll-Hypaque density centrifugation. The PBMC are resuspended in a small volume (2-5 mL) of RPMI + 10% autologous human serum, counted then diluted further with RPMI + 10% autologous human serum to 5 x 105 cells/mL. Subsequently the cells are plated in a six well Costar plastic plate precoated with 1 mg / mL fibronectin. Lipopolysaccharide, as well as the appropriate adenosine agonists and antagonists, are added simultaneously. Following incubation at 37°C for 18 hours, the cell culture supernatants are harvested, clarified and tested for TNF levels by a specific trapping ELISA.

STEP B:
ELISA for human TNFα.

A mouse anti-human TNFα monoclonal antibody is diluted to 0.5 mg/mL in PBS - $MgCl_2$ - $CaCl_2$ and added to plastic 96 - well plates. Following a 24 hr incubation at 4°C the plates are washed with PBS-Tween then treated with a solution of PBS and 1% BSA. Following additional washing with PBS Tween, aliquots of monocytes thought to contain TNFα are added to the dish, diluted to 100 mL with PBS tween and incubated for 2 hours at 37°C. The plates are further washed with PBS-Tween, then treated with a 1 to 2000 dilution of rabbit anti-human TNF polyclonal antiserum (Genzyme). The plates are incubated for 1 hour, washed then treated again with a goat anti-rabbit IgG Fab-horseradish peroxidase conjugate. The plates are incubated for one hour, washed, and the bound peroxidase is detected by the additon of a TMB peroxide mixture. TNFα levels are determined by comparison with a standard curve generated uisng pure recombinant TNFα.

EXAMPLE 15

DETECTION OF ADENOSINE RECEPTOR TRANSCRIPTS BY REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION AMPLIFICATION

STEP A:

Total RNA was extracted by the guanidinium isothiocyanate method (Chirgwin, J.M., et al, (1979) Biochemistry 18:5294-5299) from normal and LPS-stimulated human monocytes. First strand cDNA was reverse transcribed from 600 ng total RNA in a volume of 20 ml containing 20mM Tris-HCL (pH 8.4), 50mM KCl, 2.5mM $MgCl_2$, 0.1mg/ml bovine serum albumin (BSA), 0.5mM dNTP's, 10 mM DTT, 10 units SUPERSCRIPT II reverse transcriptase (LIFE TECHNOLOGIES, INC., Gathersburg, MD), and 50ng random hexamers.

STEP B:

Human adenosine receptor subtype transcript expression was determined using the polymerase chain reaction (PCR). Three µl of the randomly primed first strand cDNA, prepared from monocytes (+) or (-) LPS stimulation, was used as template in a PCR amplification reaction according to the GENEAMP protocol (PERKIN ELMER CETUS, Norwalk, CT) containing 50pmol subtype selective primers in a total volume of 100 µl. Primer pairs were designed to span four (A1 primers) and five (A2a, A2b, A3 primers) transmembrane domains and gave no or incorrect sized PCR products when tested on human genomic DNA. Primer pairs for amplification (see Table 1) were 266+267 (A1), 253+254 (A2a), 261+262 (A2b), 230+236 (A3), and 141+142 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers 141+142 are based on the published human GAPDH sequence (Tokunaga, K., et al, (1987) Cancer Research 47:5616-5619). Cycling parameters were 1 min at 94°C, 1 min at 55°C, 3 min at 72°C for 35 cycles (A1), 25 cycles (A2a), 35 cycles (A3), and 20 cycles (GAPDH). Cycling parameters for A2b were 1 min at 94°C, 1 min at 59°C, 3 min at 72°C for 30 cycles.

STEP C:

Ten µl of each PCR amplification reaction was elecrophoresed on a 1.4% agarose gel and alkaline blotted to Zeta-Probe GT membranes according to the manufacturer's protocol (BIO-RAD, Hercules, CA). Membranes were hybridized in 0.25 M sodium phosphate (pH 7.2), 0.5M NaCl, 7.0% sodium dodecyl sulphate (SDS), 1 mM EDTA, 1% BSA, and $1x10^6$ cpm/ml ^{32}P labeled probe at 50^oC. Double-stranded DNA probes were generated by Klenow enzyme extension of annealed oligonucleotide pairs including $a^{32}P$-dCTP. Oligonucleotide pairs for probe synthesis (see Table1) were 268+269 (A1), 66+67 (A2a), 263+264 (A2b), 259+260 (A3), and 143+144 (GAPDH). Oligonucleotides 259+260 are based on the published sheep A3 adenosine receptor (Linden, J., et al, (1993) Molecular Pharmacology 44:524-532) and 143+144 on the human GAPDH sequence (Tokunaga et al). Following hybridization membranes were washed to a final stringency of 75mM NaCl, 7.5mM sodium citrate, 0.1% SDS and exposed to autoradiography film. All four adenosine receptor subtypes were found to be present on monocytes through this analysis.

TABLE 1:

NAME	SEQUENCE
66	5' GCCTCTTTGAGGATGTGGTCCCCATGAACTACATGGTGTACTTCA
67	5' GCAGGGGCACCAGCACACAGGCAAAGAAGTTGAAGTACACCATGT
141	5' TCACCATCTTCCAGGAGC
142	5' ACTCCTTGGAGGCCATGT
143	5' TCCTGCACCACCAACTGCTTAGCCCCCCTGGCCAAGGTCATCCAT
144	5' CATGAGCCCTTCCACGATGCCAAAGTTGTCATGGATGACCTTGGC
230	5' GTTACCTACATCACCATG
236	5' GTTAGATAAGTTCAGACT
253	5' TCCTCGGTGTACATCACG
254	5' TCCATCTGCTTCAGCTGT
259	5' CTGGGCCTTTGCTGGCTGGTGTCATTCCTGGTGGGATTGACCCCC
260	5' TGAGGTCAGTTTCATGTTCCAGCCAAACATGGGGGTCAATCCCAC
261	5' ATGCTGCTGGAGACACAGGA
262	5' TGGTCCATCAGCTCAGTGC
263	5' GGTGGAACAGTAAAGACAGTGCCACCAACAACTGCACAGAACCCTGGGATGGAACCACGA
264	5' GGACCACATTCTCAAAGAGACACTTCACAAGGCAGCAGCTTTCATTCGTGGTTCCATCCC
266	5' CTACATCGGCATCGAGGT
267	5' GAACTCGCACTTGATCAC
268	5' TGGTGGGACTGACCCCTATGTTTGGCTGGAACAATCTGAGTGCGG
269	5' TGCTGCCGTTGGCTGCCCAGGCCCGCTCCACCGCACTCAGATTGT

While the foregoing specification teaches the principles of the present invention, with examples provided for the purpose of illustration, it will be understood that the practice of the invention encompasses all of the usual variations, adaptations, modifications, as come within the scope of the following claims and its equivalents.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Jacobson, Marlene A

(ii) TITLE OF INVENTION: INHIBITION OF TNFalpha PRODUCTION BY A2b ADENOSINE RECEPTOR AGONISTS AND ENHANCERS

(iii) NUMBER OF SEQUENCES: 56

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Merck & Co., Inc.
(B) STREET: P.O.Box 2000
(C) CITY: Rahway
(D) STATE: New Jersey
(E) COUNTRY: United States
(F) ZIP: 07065

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 6-MAY-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Bencen, Gerard H
(B) REGISTRATION NUMBER: 35,746
(C) REFERENCE/DOCKET NUMBER: 19222

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (908) 594-3901
(B) TELEFAX: (908)594-4720

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATTCGCAGCC ACGTCCTGAG GCGGCGGGAG CCCTTCAAAG CAGGTGGCAC CAGTGCCCGC        60
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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GCGGAGGCTG ATCTGCTCTC CATCACTGCC ATGAGCTGCC AAGGCGCGGG CACTGGTGCC        60
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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TCCAGAAGTT CCGGGTCACC TTCCTTAAGA TCTGGAATGA CCACTTCCGC TGCCAGCCCA        60
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGTCGTGGGG CGCCTCCTCT GGGGGGTCCT CGTCGACGGG GGGCGTGGGC TGGCAGCGGA 60

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCCTCTTTGA GGATGTGGTC CCCATGAACT ACATGGTGTA CTTCA 45

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCAGGGGCAC CAGCACACAG GCAAAGAAGT TGAAGTACAC CATGT 45

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCGCGCCGCC AGGAAGAT 18

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TATATTGAAT TCTAGACACC CAGCATGAGC 30

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCAATGGCGA TGGCCAGG 18

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TATATTGAAT TCATGGAGCT CTGCGTGAGG 30

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTAGACCATG TACTCCAT 18

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TATATTGAAT TCTGACCTTC TCGAACTCGC 30

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATTGAATTCG ATCACGGGCT CCCCCATGC 29

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATGGAGTACA TGGTCTACTT CAACTTCTTT GTGTGGGTGC TGCCCCCGCT 50

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GAAGATCCGC AAATAGACAC CCAGCATGAG CAGAAGCGGG GGCAGCACCC 50

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CCCTCTAGAG CCCAGCCTGT GCCCGCCATG CCCATCATGG GCTCC 45

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCCACCTTTT GAGCAAGTTC 20

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGCTTATGAG TATTTCTTCC 20

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 326 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Met	Pro	Pro	Ser	Ile	Ser	Ala	Phe	Gln	Ala	Ala	Tyr	Ile	Gly	Ile	Glu
1				5					10					15	
Val	Leu	Ile	Ala	Leu	Val	Ser	Val	Pro	Gly	Asn	Val	Leu	Val	Ile	Trp
			20					25					30		
Ala	Val	Lys	Val	Asn	Gln	Ala	Leu	Arg	Asp	Ala	Thr	Phe	Cys	Phe	Ile
		35					40					45			
Val	Ser	Leu	Ala	Val	Ala	Asp	Val	Ala	Val	Gly	Ala	Leu	Val	Ile	Pro
	50					55					60				
Leu	Ala	Ile	Leu	Ile	Asn	Ile	Gly	Pro	Gln	Thr	Tyr	Phe	His	Thr	Cys
65					70					75					80
Leu	Met	Val	Ala	Cys	Pro	Val	Leu	Ile	Leu	Thr	Gln	Ser	Ser	Ile	Leu
				85					90					95	
Ala	Leu	Leu	Ala	Ile	Ala	Val	Asp	Arg	Tyr	Leu	Arg	Val	Lys	Ile	Pro
			100					105					110		
Leu	Arg	Tyr	Lys	Met	Val	Val	Thr	Pro	Arg	Arg	Ala	Ala	Val	Ala	Ile
		115					120					125			

```
Ala Gly Cys Trp Ile Leu Ser Phe Val Val Gly Leu Thr Pro Met Phe
    130                 135                 140

Gly Trp Asn Asn Leu Ser Ala Val Glu Arg Ala Trp Ala Ala Asn Gly
145                 150                 155                 160

Ser Met Gly Glu Pro Val Ile Lys Cys Glu Phe Glu Lys Val Ile Ser
                165                 170                 175

Met Glu Tyr Met Val Tyr Phe Asn Phe Phe Val Trp Val Leu Pro Pro
            180                 185                 190

Leu Leu Leu Met Val Leu Ile Tyr Leu Glu Val Phe Tyr Leu Ile Arg
        195                 200                 205

Lys Gln Leu Asn Lys Lys Val Ser Ala Ser Ser Gly Asp Pro Gln Lys
    210                 215                 220

Tyr Tyr Gly Lys Glu Leu Lys Ile Ala Lys Ser Leu Ala Leu Ile Leu
225                 230                 235                 240

Phe Leu Phe Ala Leu Ser Trp Leu Pro Leu His Ile Leu Asn Cys Ile
                245                 250                 255

Thr Leu Phe Cys Pro Ser Cys His Lys Pro Ser Ile Leu Thr Tyr Ile
            260                 265                 270

Ala Ile Phe Leu Thr His Gly Asn Ser Ala Met Asn Pro Ile Val Tyr
        275                 280                 285

Ala Phe Arg Ile Gln Lys Phe Arg Val Thr Phe Leu Lys Ile Trp Asn
    290                 295                 300

Asp His Phe Arg Cys Gln Pro Ala Pro Pro Ile Asp Glu Asp Leu Pro
305                 310                 315                 320

Glu Glu Arg Pro Asp Asp
                325
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(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 981 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

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ATGCCGCCCT CCATCTCAGC TTTCCAGGCC GCCTACATCG GCATCGAGGT GCTCATCGCC     60
CTGGTCTCTG TGCCCGGGAA CGTGCTGGTG ATCTGGGCGG TGAAGGTGAA CCAGGCGCTG    120
CGGGATGCCA CCTTCTGCTT CATCGTGTCG CTGGCGGTGG CTGATGTGGC CGTGGGTGCC    180
CTGGTCATCC CCCTCGCCAT CCTCATCAAC ATTGGGCCAC AGACCTACTT CCACACCTGC    240
CTCATGGTTG CCTGTCCGGT CCTCATCCTC ACCCAGAGCT CCATCCTGGC CCTGCTGGCA    300
ATTGCTGTGG ACCGCTACCT CCGGGTCAAG ATCCCTCTCC GGTACAAGAT GGTGGTGACC    360
CCCCGGAGGG CGGCGGTGGC CATAGCCGGC TGCTGGATCC TCTCCTTCGT GGTGGGACTG    420
ACCCCTATGT TTGGCTGGAA CAATCTGAGT GCGGTGGAGC GGGCCTGGGC AGCCAACGGC    480
AGCATGGGGG AGCCCGTGAT CAAGTGCGAG TTCGAGAAGG TCATCAGCAT GGAGTACATG    540
GTCTACTTCA ACTTCTTTGT GTGGGTGCTG CCCCCGCTTC TCCTCATGGT CCTCATCTAC    600
CTGGAGGTCT TCTACCTAAT CCGCAAGCAG CTCAACAAGA AGGTGTCGGC CTCCTCCGGC    660
GACCCGCAGA AGTACTATGG GAAGGAGCTG AAGATCGCCA AGTCGCTGGC CCTCATCCTC    720
TTCCTCTTTG CCCTCAGCTG GCTGCCTTTG CACATCCTCA ACTGCATCAC CCTCTTCTGC    780
CCGTCCTGCC ACAAGCCCAG CATCCTTACC TACATTGCCA TCTTCCTCAC GCACGGCAAC    840
TCGGCCATGA ACCCCATTGT CTATGCCTTC CGCATCCAGA AGTTCCGCGT CACCTTCCTT    900
AAGATTTGGA ATGACCATTT CCGCTGCCAG CCTGCACCTC CCATTGACGA GGATCTCCCA    960
GAAGAGAGGC CTGATGACTA G                                              981
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 412 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Met Pro Ile Met Gly Ser Ser Val Tyr Ile Thr Val Glu Leu Ala Ile
1               5                   10                  15
```

```
Ala Val Leu Ala Ile Leu Gly Asn Val Leu Val Cys Trp Ala Val Trp
            20                  25                  30

Leu Asn Ser Asn Leu Gln Asn Val Thr Asn Tyr Phe Val Val Ser Leu
        35                  40                  45

Ala Ala Ala Asp Ile Ala Val Gly Val Leu Ala Ile Pro Phe Ala Ile
    50                  55                  60

Thr Ile Ser Thr Gly Phe Cys Ala Ala Cys His Gly Cys Leu Phe Ile
65                  70                  75                  80

Ala Cys Phe Val Leu Val Leu Thr Gln Ser Ser Ile Phe Ser Leu Leu
                85                  90                  95

Ala Ile Ala Ile Asp Arg Tyr Ile Ala Ile Arg Ile Pro Leu Arg Tyr
            100                 105                 110

Asn Gly Leu Val Thr Gly Thr Arg Ala Lys Gly Ile Ile Ala Ile Cys
        115                 120                 125

Trp Val Leu Ser Phe Ala Ile Gly Leu Thr Pro Met Leu Gly Trp Asn
    130                 135                 140

Asn Cys Gly Gln Pro Lys Glu Gly Lys Asn His Ser Gln Gly Cys Gly
145                 150                 155                 160

Glu Gly Gln Val Ala Cys Leu Phe Glu Asp Val Val Pro Met Asn Tyr
                165                 170                 175

Met Val Tyr Phe Asn Phe Phe Ala Cys Val Leu Val Pro Leu Leu Leu
            180                 185                 190

Met Leu Gly Val Tyr Leu Arg Ile Phe Leu Ala Ala Arg Arg Gln Leu
        195                 200                 205

Lys Gln Met Glu Ser Gln Pro Leu Pro Gly Glu Arg Ala Arg Ser Thr
    210                 215                 220

Leu Gln Lys Glu Val His Ala Ala Lys Ser Leu Ala Ile Ile Val Gly
225                 230                 235                 240

Leu Phe Ala Leu Cys Trp Leu Pro Leu His Ile Ile Asn Cys Phe Thr
                245                 250                 255

Phe Phe Cys Pro Asp Cys Ser His Ala Pro Leu Trp Leu Met Tyr Leu
            260                 265                 270

Ala Ile Val Leu Ser His Thr Asn Ser Val Val Asn Pro Phe Ile Tyr
        275                 280                 285

Ala Tyr Arg Ile Arg Glu Phe Arg Gln Thr Phe Arg Lys Ile Ile Arg
    290                 295                 300

Ser His Val Leu Arg Gln Gln Glu Pro Phe Lys Ala Ala Gly Thr Ser
```

305 310 315 320

Ala Arg Val Leu Ala Ala His Gly Ser Asp Gly Glu Gln Val Ser Leu
325 330 335

Arg Leu Asn Gly His Pro Pro Gly Val Trp Ala Asn Gly Ser Ala Pro
340 345 350

His Pro Glu Arg Arg Pro Asn Gly Tyr Ala Leu Gly Leu Val Ser Gly
355 360 365

Gly Ser Ala Gln Glu Ser Gln Gly Asn Thr Gly Leu Pro Asp Val Glu
370 375 380

Leu Leu Ser His Glu Leu Lys Gly Val Cys Pro Glu Pro Pro Gly Leu
385 390 395 400

Asp Asp Pro Leu Ala Gln Asp Gly Ala Gly Val Ser
405 410

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1239 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATGCCCATCA TGGGCTCCTC GGTGTACATC ACGGTGGAGC TGGCCATTGC TGTGCTGGCC 60

ATCCTGGGCA ATGTGCTGGT GTGCTGGGCC GTGTGGCTCA ACAGCAACCT GCAGAACGTC 120

ACCAACTACT TTGTGGTGTC ACTGGCGGCG GCCGACATCG CAGTGGGTGT GCTCGCCATC 180

CCCTTTGCCA TCACCATCAG CACCGGGTTC TGCGCTGCCT GCCACGGCTG CCTCTTCATT 240

GCCTGCTTCG TCCTGGTCCT CACGCAGAGC TCCATCTTCA GTCTCCTGGC CATCGCCATT 300

GACCGCTACA TTGCCATCCG CATCCCGCTC CGGTACAATG GCTTGGTGAC CGGCACGAGG 360

GCTAAGGGCA TCATTGCCAT CTGCTGGGTG CTGTCGTTTG CCATCGGCCT GACTCCCATG 420

CTAGGTTGGA ACAACTGCGG TCAGCCAAAG GAGGGCAAGA ACCACTCCCA GGGCTGCGGG 480

GAGGGCCAAG TGGCCTGTCT CTTTGAGGAT GTGGTCCCCA TGAACTACAT GGTGTACTTC 540

AACTTCTTTG CCTGTGTGCT GGTGCCCCTG CTGCTCATGC TGGGTGTCTA TTTGCGGATC 600

TTCCTGGCGG CGCGACGACA GCTGAAGCAG ATGGAGAGCC AGCCTCTGCC GGGGGAGCGG 660

GCACGGTCCA CACTGCAGAA GGAGGTCCAT GCTGCCAAGT CACTGGCCAT CATTGTGGGG 720

CTCTTTGCCC TCTGCTGGCT GCCCCTACAC ATCATCAACT GCTTCACTTT CTTCTGCCCC 780

GACTGCAGCC ACGCCCCTCT CTGGCTCATG TACCTGGCCA TCGTCCTCTC CCACACCAAT 840

TCGGTTGTGA ATCCCTTCAT CTACGCCTAC CGTATCCGCG AGTTCCGCCA GACCTTCCGC 900

AAGATCATTC GCAGCCACGT CCTGAGGCAG CAAGAACCTT TCAAGGCAGC TGGCACCAGT 960

GCCCGGGTCT TGGCAGCTCA TGGCAGTGAC GGAGAGCAGG TCAGCCTCCG TCTCAACGGC 1020

CACCCGCCAG GAGTGTGGGC CAACGGCAGT GCTCCCCACC CTGAGCGGAG GCCCAATGGC 1080

TATGCCCTGG GGCTGGTGAG TGGAGGGAGT GCCCAAGAGT CCCAGGGGAA CACGGGCCTC 1140

CCAGACGTGG AGCTCCTTAG CCATGAGCTC AAGGGAGTGT GCCCAGAGCC CCCTGGCCTA 1200

GATGACCCCC TGGCCCAGGA TGGAGCAGGA GTGTCCTGA 1239

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 332 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 216
(D) OTHER INFORMATION: /label= threonine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met Leu Leu Glu Thr Gln Asp Ala Leu Tyr Val Ala Leu Glu Leu Val
1   5   10   15

Ile Ala Ala Leu Ser Val Ala Gly Asn Val Leu Val Cys Ala Ala Val
            20   25   30

Gly Thr Ala Asn Thr Leu Gln Thr Pro Thr Asn Tyr Phe Leu Val Ser
        35   40   45

Leu Ala Ala Ala Asp Val Ala Val Gly Leu Phe Ala Ile Pro Phe Ala
50 55 60

Ile Thr Ile Ser Leu Gly Phe Cys Thr Asp Phe Tyr Gly Cys Leu Phe
65 70 75 80

Leu Ala Cys Phe Val Leu Val Leu Thr Gln Ser Ser Ile Phe Ser Leu
85 90 95

Leu Ala Val Ala Val Asp Arg Tyr Leu Ala Ile Cys Val Pro Leu Arg
100 105 110

Tyr Lys Ser Leu Val Thr Gly Thr Arg Ala Arg Gly Val Ile Ala Val
115 120 125

Leu Trp Val Leu Ala Phe Gly Ile Gly Leu Thr Pro Phe Leu Gly Trp
130 135 140

Asn Ser Lys Asp Ser Ala Thr Asn Asn Cys Thr Glu Pro Trp Asp Gly
145 150 155 160

Thr Thr Asn Glu Ser Cys Cys Leu Val Lys Cys Leu Phe Glu Asn Val
165 170 175

Val Pro Met Ser Tyr Met Val Tyr Phe Asn Phe Phe Gly Cys Val Leu
180 185 190

Pro Pro Leu Leu Ile Met Leu Val Ile Tyr Ile Lys Ile Phe Leu Val
195 200 205

Ala Cys Arg Gln Leu Gln Arg Xaa Glu Leu Met Asp His Ser Arg Thr
210 215 220

Thr Leu Gln Arg Glu Ile His Ala Ala Lys Ser Leu Ala Met Ile Val
225 230 235 240

Gly Ile Phe Ala Leu Cys Trp Leu Pro Val His Ala Val Asn Cys Val
245 250 255

Thr Leu Phe Gln Pro Ala Gln Gly Lys Asn Lys Pro Lys Trp Ala Met
260 265 270

Asn Met Ala Ile Leu Leu Ser His Ala Asn Ser Val Val Asn Pro Ile
275 280 285

Val Tyr Ala Tyr Arg Asn Arg Asp Phe Arg Tyr Thr Phe His Lys Ile
290 295 300

Ile Ser Arg Tyr Leu Leu Cys Gln Ala Asp Val Lys Ser Gly Asn Gly
305 310 315 320

Gln Ala Gly Val Gln Pro Ala Leu Gly Val Gly Leu
325 330

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 999 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
ATGCTGCTGG AGACACAGGA CGCGCTGTAC GTGGCGCTGG AGCTGGTCAT CGCCGCGCTT     60
TCGGTGGCGG GCAACGTGCT GGTGTGCGCC GCGGTGGGCA CGGCGAACAC TCTGCAGACG    120
CCCACCAACT ACTTCCTGGT GTCCCTGGCT GCGGCCGACG TGGCCGTGGG GCTCTTCGCC    180
ATCCCCTTTG CCATCACCAT CAGCCTGGGC TTCTGCACTG ACTTCTACGG CTGCCTCTTC    240
CTCGCCTGCT TCGTGCTGGT GCTCACGCAG AGCTCCATCT TCAGCCTTCT GGCCGTGGCA    300
GTCGACAGAT ACCTGGCCAT CTGTGTCCCG CTCAGGTATA AAAGTTTGGT CACGGGGACC    360
CGAGCAAGAG GGGTCATTGC TGTCCTCTGG GTCCTTGCCT TTGGCATCGG ATTGACTCCA    420
TTCCTGGGGT GGAACAGTAA AGACAGTGCC ACCAACAACT GCACAGAACC CTGGGATGGA    480
ACCACGAATG AAAGCTGCTG CCTTGTGAAG TGTCTCTTTG AGAATGTGGT CCCCATGAGC    540
TACATGGTAT ATTTCAATTT CTTTGGGTGT GTTCTGCCCC CACTGCTTAT AATGCTGGTG    600
ATCTACATTA AGATCTTCCT GGTGGCCTGC AGGCAGCTTC AGCGCACTGA GCTGATGGAC    660
CACTCGAGGA CCACCCTCCA GCGGGAGATC CATGCAGCCA AGTCACTGGC CATGATTGTG    720
GGGATTTTTG CCCTGTGCTG GTTACCTGTG CATGCTGTTA ACTGTGTCAC TCTTTTCCAG    780
CCAGCTCAGG GTAAAAATAA GCCCAAGTGG GCAATGAATA TGGCCATTCT TCTGTCACAT    840
GCCAATTCAG TTGTCAATCC CATTGTCTAT GCTTACCGGA ACCGAGACTT CCGCTACACT    900
TTTCACAAAA TTATCTCCAG GTATCTTCTC TGCCAAGCAG ATGTCAAGAG TGGGAATGGT    960
CAGGCTGGGG TACAGCCTGC TCTCGGTGTG GGCCTATGA                           999
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 318 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
Met Pro Asn Asn Ser Thr Ala Leu Ser Leu Ala Asn Val Thr Tyr Ile
1               5                   10                  15

Thr Met Glu Ile Phe Ile Gly Leu Cys Ala Ile Val Gly Asn Val Leu
            20                  25                  30

Val Ile Cys Val Val Lys Leu Asn Pro Ser Leu Gln Thr Thr Thr Phe
        35                  40                  45

Tyr Phe Ile Val Ser Leu Ala Leu Ala Asp Ile Ala Val Gly Val Leu
    50                  55                  60

Val Met Pro Leu Ala Ile Val Val Ser Leu Gly Ile Thr Ile His Phe
65                  70                  75                  80

Tyr Ser Cys Leu Phe Met Thr Cys Leu Leu Leu Ile Phe Thr His Ala
                85                  90                  95

Ser Ile Met Ser Leu Leu Ala Ile Ala Val Asp Arg Tyr Leu Arg Val
            100                 105                 110

Lys Leu Thr Val Arg Tyr Lys Arg Val Thr Thr His Arg Arg Ile Trp
        115                 120                 125

Leu Ala Leu Gly Leu Cys Trp Leu Val Ser Phe Leu Val Gly Leu Thr
    130                 135                 140

Pro Met Phe Gly Trp Asn Met Lys Leu Thr Ser Glu Tyr His Arg Asn
145                 150                 155                 160

Val Thr Phe Leu Ser Cys Gln Phe Val Ser Val Met Arg Met Asp Tyr
                165                 170                 175

Met Val Tyr Phe Ser Phe Leu Thr Trp Ile Phe Ile Pro Leu Val Val
            180                 185                 190

Met Cys Ala Ile Tyr Leu Asp Ile Phe Tyr Ile Ile Arg Asn Lys Leu
        195                 200                 205

Ser Leu Asn Leu Ser Asn Ser Lys Glu Thr Gly Ala Phe Tyr Gly Arg
    210                 215                 220
```

```
Glu Phe Lys Thr Ala Lys Ser Leu Phe Leu Val Leu Phe Leu Phe Ala
225                 230                 235                 240

Leu Ser Trp Leu Pro Leu Ser Ile Ile Asn Cys Ile Ile Tyr Phe Asn
                245                 250                 255

Gly Glu Val Pro Gln Leu Val Leu Tyr Met Gly Ile Leu Leu Ser His
            260                 265                 270

Ala Asn Ser Met Met Asn Pro Ile Val Tyr Ala Tyr Lys Ile Lys Lys
        275                 280                 285

Phe Lys Glu Thr Tyr Leu Leu Ile Leu Lys Ala Cys Val Val Cys His
    290                 295                 300

Pro Ser Asp Ser Leu Asp Thr Ser Ile Glu Lys Asn Ser Glu
305                 310                 315
```

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 957 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
ATGCCCAACA ACAGCACTGC TCTGTCATTG GCCAATGTTA CCTACATCAC CATGGAAATT    60
TTCATTGGAC TCTGCGCCAT AGTGGGCAAC GTGCTGGTCA TCTGCGTGGT CAAGCTGAAC   120
CCCAGCCTGC AGACCACCAC CTTCTATTTC ATTGTCTCTC TAGCCCTGGC TGACATTGCT   180
GTTGGGGTGC TGGTCATGCC TTTGGCCATT GTTGTCAGCC TGGGCATCAC AATCCACTTC   240
TACAGCTGCC TTTTTATGAC TTGCCTACTG CTTATCTTTA CCCACGCCTC CATCATGTCC   300
TTGCTGGCCA TCGCTGTGGA CCGATACTTG CGGGTCAAGC TTACCGTCAG ATACAAGAGG   360
GTCACCACTC ACAGAAGAAT ATGGCTGGCC CTGGGCCTTT GCTGGCTGGT GTCATTCCTG   420
GTGGGATTGA CCCCCATGTT TGGCTGGAAC ATGAAACTGA CCTCAGAGTA CCACAGAAAT   480
GTCACCTTCC TTTCATGCCA ATTTGTTTCC GTCATGAGAA TGGACTACAT GGTATACTTC   540
AGCTTCCTCA CCTGGATTTT CATCCCCCTG GTTGTCATGT GCGCCATCTA TCTTGACATC   600
```

TTTTACATCA TTCGGAACAA ACTCAGTCTG AACTTATCTA ACTCCAAAGA GACAGGTGCA 660

TTTTATGGAC GGGAGTTCAA GACGGCTAAG TCCTTGTTTC TGGTTCTTTT CTTGTTTGCT 720

CTGTCATGGC TGCCTTTATC TATCATCAAC TGCATCATCT ACTTTAATGG TGAGGTACCA 780

CAGCTTGTGC TGTACATGGG CATCCTGCTG TCCCATGCCA ACTCCATGAT GAACCCTATC 840

GTCTATGCCT ATAAAATAAA GAAGTTCAAG GAAACCTACC TTTTGATCCT CAAAGCCTGT 900

GTGGTCTGCC ATCCCTCTGA TTCTTTGGAC ACAAGCATTG AGAAGAATTC TGAGTAG 957

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCCAAGCTTA TGAAAGCCAA CAATACC 27

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TGCTCTAGAC TCTGGTATCT TCACATT 27

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GCCTCTTTGA GGATGTGGTC CCCATGAACT ACATGGTGTA CTTCA 45

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GCAGGGGCAC CAGCACACAG GCAAAGAAGT TGAAGTACAC CATGT 45

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TCACCATCTT CCAGGAGC 18

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

ACTCCTTGGA GGCCATGT 18

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TCCTGCACCA CCAACTGCTT AGCCCCCCTG GCCAAGGTCA TCCAT 45

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CATGAGCCCT TCCACGATGC CAAAGTTGTC ATGGATGACC TTGGC 45

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GTTACCTACA TCACCATG 18

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GTTAGATAAG TTCAGACT 18

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CTGACCTCAG AGTACCACAG AAATGTCACC TTCCTTTCAT GCCAA 45

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TTGGCATGAA AGGAAGGTGA CATTTCTGTG GTACTCTGAG GTCAG 45

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CTCAGTCTGA ACTTATCTAA CTCCAAAGAG ACAGGTGCAT TTTATG 46

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CATAAAATGC ACCTGTCTCT TTGGAGTTAG ATAAGTTCAG ACTGAG 46

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

TCCTCGGTGT ACATCACG 18

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

TCCATCTGCT TCAGCTGT 18

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CTGGGCCTTT GCTGGCTGGT GTCATTCCTG GTGGGATTGA CCCCC 45

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

TGAGGTCAGT TTCATGTTCC AGCCAAACAT GGGGGTCAAT CCCAC 45

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

ATGCTGCTGG AGACACAGGA 20

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

TGGTCCATCA GCTCAGTGC 19

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GGTGGAACAG TAAAGACAGT GCCACCAACA ACTGCACAGA ACCCTGGGAT GGAACCACGA 60

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GGACCACATT CTCAAAGAGA CACTTCACAA GGCAGCAGCT TTCATTCGTG GTTCCATCCC 60

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CTACATCGGC ATCGAGGT 18

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

GAACTCGCAC TTGATCAC 18

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

TGGTGGGACT GACCCCTATG TTTGGCTGGA ACAATCTGAG TGCGG 45

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

TGCTGCCGTT GGCTGCCCAG GCCCGCTCCA CCGCACTCAG ATTGT 45

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

CTGAGCTCAG CAGACGAAAA CCTCACCTTC CTACCCTGCC GA 42

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

TCGGCAGGGT AGGAAGGTGA GGTTTTCGTC TGCTGAGCTC AG 42

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

CTCAGCCAGA GCTTTTCTGG CTCCAGAGAG ACAGGCGCAT TCTATG 46

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

CATAGAATGC GCCTGTCTCT CTGGAGCCAG AAAAGCTCTG GCTGAG 46

WHAT IS CLAIMED IS:

1. A method for inhibiting TNFα production which comprises contacting the A2b subtype of the adenosine receptor with an adenosine receptor agonist.

2. A method for treating or preventing autoimmune diseases including rheumatoid arthritis, rheumatoid spondylitis, inflammatory bowl disease (ulcerative colitis and Crohns disease), intestinal pathology associated with graft vs. host disease, organ transplant reactions, septic shock, fever and myalgia due to infection and cachexia associated with chronic infections, malignancy and aquired immune deficiency syndrome, pulmonary diseases such as pulmonary sarcoidosis, silicosis, chronic pulmonary inflammatory disease, adult respiratory distress syndrome which comprises providing a sufficient quantity of an A2b adenosine receptor agonist to inhibit TNFα production.

3. A method for increasing cAMP accumulation in monocytes, and thereby inhibiting production of TNFα, which comprises contacting the monocyte A2b adenosine receptor subtype with an adenosine receptor agonist at a sufficient concentration to activate adenylate cyclase.

4. The method of any one of claims 1, 2, 3, or 4, wherein the adenosine receptor agonist is adenosine, CPCA, NECA, R-PIA, or CHA.

5. A method for inhibiting TNFα production which comprises contacting the A2b subtype of the adenosine receptor with an A2b adenosine receptor enhancer.

6. A method for identifying A2b adenosine receptor agonist enhancer or A2b receptor selective compounds which comprises the steps of:

(a) contacting monocytes with a test compound and measuring the effect of the test compound on TNFα production;

(b) contacting a test compound, identified according to step (a) as inhibiting TNFα production by the monocytes, with membranes derived from a stable cell line individually expressing each of the A1, A2a, A2b, or A3 adenosine receptor or with the whole cell expressing each of the A1, A2a, A2b, or A3 adenosine receptor and measuring the binding affinity of the test compound for the receptor or the effect of the test compound on cAMP production in the stable cell line;

(c) selecting compounds which bind to the A2b adenosine receptor or which induces elevation in cAMP in the cell line expressing the A2b adenosine receptor and which do not bind to membranes or affect the cAMP level in the stable cell lines expressing the A1, A2a, or A3 adenosine receptor subtypes.

7. A method for inhibiting production of TNFα by activated monocytes which comprises contacting monocytes with an inhibitorily effective amount of a compound identified according to Claim 6.

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Patents Act 1977 **Examiner's report to the Comptroller under Section 17** **(The Search report)**	Application number GB 9508844.9
Relevant Technical Fields (i) UK Cl (Ed.N) A5B (BHA) (ii) Int Cl (Ed.6) A61K 31/52	Search Examiner C SHERRINGTON Date of completion of Search 16 AUGUST 1995
Databases (see below) (i) UK Patent Office collections of GB, EP, WO and US patent specifications. (ii) ONLINE: WPI, CLAIMS, DIALOG/BIOTECH	Documents considered relevant following a search in respect of Claims :- 1, 3 to 7

Categories of documents

- **X:** Document indicating lack of novelty or of inventive step.
- **Y:** Document indicating lack of inventive step if combined with one or more other documents of the same category.
- **A:** Document indicating technological background and/or state of the art.
- **P:** Document published on or after the declared priority date but before the filing date of the present application.
- **E:** Patent document published on or after, but with priority date earlier than, the filing date of the present application.
- **&:** Member of the same patent family; corresponding document.

Category	Identity of document and relevant passages	Relevant to claim(s)
X	GB 2264948 A (MERCK & CO INC) whole document, especially Table L, page 8, line 25; page 11, line 7 to page 13, line 13	1, 3 to 7
X	WO 93/25677 A1 (GARVAN INSTITUTE OF MEDICAL RESEARCH) whole document, especially page 3, lines 17 to 31, Claim 7; Figure 4B	1, 3 to 7
X	US Pat. Appl. NTIS US 7-577528 especially pages 40 to 64; Figure 1	1, 3 to 5
A	Life Sci. 1993, 52, 1917-1924 Inhibition of human monocyte TNF production by adenosime receptor agonists	1, 4, 5
X	Biochem. Biophys. Res. Commun. 1992, 187(1), 86-93 Molecular Cloning and Expression of an Adenosine A2b Receptor from Human Brain	1, 3 to 7
A	Mol. Endocrinol. 1992, 6, 384-393 Molecular Cloning and Expression of the cDNA for a Novel A2-Adenosine Receptor Subtype	1

Databases: The UK Patent Office database comprises classified collections of GB, EP, WO and US patent specifications as outlined periodically in the Official Journal (Patents). The on-line databases considered for search are also listed periodically in the Official Journal (Patents).